Dorsal-ventral patterning and the control of neural cell fate in the vertebrate ventral neural tube

Thesis

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Dorsal-ventral patterning and the control of Neural Cell Fate in the Vertebrate Ventral Neural Tube

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Αφιερωμένο στους γονείς μου

Dedicated to my parents

You will do some foolish things, but do them with enthusiasm!

Colette

Abstract

During embryonic development organised structures are formed from homogeneous groups of undifferentiated cells. The specification of distinct cell types in these tissues is believed to be controlled by molecular cues that determine a cell's fate according to the position of the cell within the tissue. In ventral regions of the vertebrate spinal cord the secreted protein Sonic Hedgehog (Shh) has been characterised as one such molecular cue. Shh appears to act at distance from its source, in a concentration dependent manner, to control the dorsal-ventral position in which distinct neuronal subtypes are generated. In this study I focus on the mechanisms of developmental patterning by graded Hedgehog (Hh) signalling that control the patterning of the spinal cord.

Characterisation of the expression pattern of a series of molecular markers indicates that dorsal ventral patterning of the zebrafish spinal cord is similar to the mouse and the chick. Furthermore, use of the selective Hh signalling antagonist, cyclopamine, indicated that in zebrafish expression profiles of these molecular markers is dependent on Hh signalling. Using this approach, we provide evidence that ventral neural patterning depends on both the strength and duration of Hh signal *in vivo*. These data provide *in vivo* support for the idea that a gradient of Hh signalling is responsible for providing positional information to the ventral neural tube.

To further understand how graded Hh signalling is interpreted by cells in the ventral neural tube we have analysed the spinal cord and hindbrain of mouse embryos lacking two genes, Nkx2.2 and Pax6, important for ventral vertebrate neural patterning. Previous analysis of each single mutant ($Nkx2.2^{-/-}$ and $Pax6^{-/-}$)

suggested a model in which Nkx2.2 is required for the generation of the most ventral interneuron type in the spinal cord, V3 neurons, while Pax6 is required to limit the expression of Nkx2.2. Analysis of mutant embryos lacking both Nkx2.2 and Pax6 has led us to modify this model. Our data indicate that Nkx2.2 is not directly required for V3 neuron generation but instead is required to repress Olig2 or similarly expressed gene. Moreover, our analysis revealed that Olig2 is regulated differently in the hindbrain and spinal cord and these data are consistent with the idea that Nkx2.2 is required for the generation of hindbrain visceral motor neurons. Finally, analysis of Ngn3 mutant mice indicated that Nkx2.2 acts upstream of Ngn3 and that Ngn3 is not required for V3 neuron generation.

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Abbreviations

A-P	Anterior-posterior
ash	Achaete-scute
bHLH	Basic helix-loop-helix
BMPs	Bone morphogenetic proteins
BSA	Bovine serum albumin
Ci	Cubitus interruptus
CiR	Cubitus interruptus repressor form
CNS	Central nervous system
cyc	Cyclops
DAPI	4'-6-Diamidino-2-phenylindole
Dhh	Desert hedgehog
dpc	Days *post coitum*
dtr	Detour
D-V	Dorsal-ventral
E	Embryonic day
EDTA	Ethylenediaminotetraacetic
Ehh	Echidna hedgehog
FGF	Fibroblast Growth Factor
FITC	Fluorescein isothiocyanate
FP	Floor plate
Fu	Fused
g	Grams

gp	Guinea pig
HD	Homeodomain
Hh	Hedgehog
hMNs	Hypoglossal motor neurons
hpf	Hours post fertilisation
Ihh	Indian hedgehog
λ	Wavelength
l	Litre
LFP	Lateral floor plate
m	Mouse
MFP	Medial floor plate
M	Molar
mg	Milligram
ml	Millilitre
µl	Microlitre
µm/uM	Micromolar
mM	Millimolar
MNs	Motor neurons
NC	Notochord
ng	Nanograms
nm	Nanometre
nM	Nanomolar
p	Progenitors
p0	Progenitors of V0 interneurons
p1	Progenitors of V1 interneurons

p2	Progenitors of V2 interneurons
p3	Progenitors of V3 interneurons
PCR	Polymerase chain reaction
PB	Phosphate Buffer
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PKA	Cyclic adenosine monophosphate (cAMP) dependent protein kinase
pMN	Progenitors of motor neurons
Ptc	Patched
r	Rabbit
SC	Spinal cord
Shh	Sonic hedgehog
sMNs	Somatic motor neurons
Smo	Smoothened
syu	sonic you
TRIS	Tris[hydroxymethyl]aminomethane
Twhh	Tiggy winkle hedgehog
vMNs	Visceral motor neurons
v/v	Volume/volume
wg	Wingless gene
Wnts	Wingless
WT	wild type
w/v	Weight/volume
Xt	Extra toes
z	Zebrafish

1. Introduction

Development is the formation of organised structures from homogeneous groups of undifferentiated cells. It involves five distinct processes, namely, cell division, pattern formation, cell differentiation and growth. The development of most tissues involves progressive stages in which the cells that comprise the tissue acquire increasingly precise identity. Thousands of genes are involved in controlling the complex process in animals and plants and even though significant progress has been made over the last century, still many unanswered questions remain.

Although there is great diversity between different animals, development of most of them proceeds through a number of common stages. Once the fundamental developmental processes have occurred all vertebrates go through a similar *phylotypic stage* showing the characteristic features of chordate embryos: the notochord, somites and the neural tube (Twyman, 2001; Wolpert et al.., 2002). Post-phylotypic development is responsible for evolutionary diversification within phyla which will eventually distinguish an individual animal from others.

It is increasingly clear that animals within a phylum and even in different phyla use similar molecular mechanisms during the various developmental stages. As development proceeds cells acquire unique position and identity that determines their fate. This positional information corresponds to molecular cues that consist of extracellular signals and the intracellular responses to these signals. It is evident that the same families of signals are frequently used in different species to accomplish similar tasks.

The major aim of this thesis is to advance our understanding of the mechanisms involved in regulating the development of the central nervous system (CNS) and for that mouse and zebrafish have been used as model organisms.

1.1 Mouse and Zebrafish Model Systems

Although different species acquire different final forms, the presence of the phylotypic stage as well as the sharing of molecular mechanisms means that vertebrates share a number of common features and can be used "interchangeably". For this reason, mouse, chicken, zebrafish and *Xenopus* embryos have been used extensively in developmental neurobiology.

To allow the correct use of the various animals as developmental models an important issue is "developmental staging". Development is normally monitored by looking at pre-specified stages rather than time of fertilisation. This is to avoid any possible effect caused by external and environmental factors. Staging is by reference to the structure of the embryo, for example the number of somites formed. Prior to somite development other embryonic features are used and for mouse developmental time is expressed as days *post coitum* (dpc) or embryonic day (E) meaning days after mating (Twyman, 2001; Wolpert et al.., 2002). Staging of zebrafish embryos is also by reference to embryologically visible events specified by Kimmel *at al* (Kimmel et al.., 1995). Due to the more rapid development timing is generally referred to as hours post fertilisation (hpf) (Kimmel et al.., 1995).

The choice amongst different animal models during developmental studies depends on the techniques available as well as how extensively studied and understood an animal model is. An advantage of using mouse is that is closely related to human development. Additionally, from fertilisation to mature adult it has a relatively short life cycle of 9 weeks. This feature, along with the well established genetic knowledge of the mouse genome and the easy generation of mutants by genetic modification have made the mouse an attractive and commonly used model for developmental studies. Particularly important is also the

ability to create mutant mice using reverse genetics in order to analyse the function of particular genes (Landel et al.., 1990; Tronche et al.., 2002). However, like all mammals the embryo develops inside the mother, which makes it difficult to access for experimental manipulation or continuous observation. It is possible for mouse embryos to be cultured outside the mother but they are only viable for short periods of time (Wolpert et al.., 2002). Moreover, the litter size is small and it requires sacrifice of the mother.

Over the past decade the zebrafish (*Danio rerio*) has received a lot of attention and has become a particularly popular model organism for the study of vertebrate developmental biology (Lewis and Eisen, 2003; Udvadia and Linney, 2003). Zebrafish offer several advantages as a model organism including low maintenance costs, a short life cycle of approximately 12 weeks (only slightly longer than the mouse life cycle), large number of embryos obtained from individual females at regular basis all year round and most important translucent embryos (Lewis and Eisen, 2003; Udvadia and Linney, 2003). The short life cycle of zebrafish embryos allows genetic studies to be carried out easily and on a regular basis, while the optical clarity of the embryos, which develop entirely outside the mother, allows visualisation of individual cells within the embryos throughout development (Lewis and Eisen, 2003; Udvadia and Linney, 2003; Wolpert et al.., 2002). Additionally, the development of a number of genetic tools that can be used when studying zebrafish development have generated important resources for the in depth understanding of various developmental questions. These genetic tools include anti-sense morpholino technology, gain of function RNA injections, the isolation of a number of mutations and the generation of stable transgenic lines (Lewis and Eisen, 2003; Udvadia and Linney, 2003).

1.1.1 Overview of mouse and zebrafish embryology

Development of both mouse and zebrafish begins with fertilisation where a diploid cell, the zygote, is generated. Fertilisation is followed by a series of distinct events that happen at precise time and order to eventually give rise to a developed multicellular organism. These events are: cleavage, gastrulation, neurulation and organogenesis. In all animal embryos, cleavage, a series of rapid and synchronous cell divisions, results in the formation of a number of smaller cells termed blastomeres (Twyman, 2001). Different names are used to describe this stage at different species such as blastodisc in fish and blastocyst in mammals (Twyman, 2001). Gastrulation involves a series of complex cell movements that reorganises the embryo into three germ layers (ectoderm, mesoderm and endoderm) and is followed by neurulation, the development of the central nervous system (Twyman, 2001). Organogenesis, the formation of individual organs, follows the basic developmental processes (Twyman, 2001).

1.1.2 Mouse

In the mouse, fertilisation of the egg takes place in the oviduct, where cleavage also happens, approximately 5 days later. Gastrulation begins 6.5dpc with the formation of the primitive streak in the embryonic epiblast (Twyman, 2001). In the next 24 hours the streak elongates and at the anterior end of the streak, a specialized structure forms, which is known as the node (Beddington and Robertson, 1999). Proliferating epiblast cells move through the streak and differentiate as mesoderm and endoderm (Twyman, 2001). One of the earliest mesodermal structures is the notochord, a rod-shaped structure, which forms along the anterior-posterior body axis (Wolpert et al.., 2002). Blocks of mesodermal tissue will form the somites on either side of the notochord which will in turn induce the

formation of the vertebral column and the muscles of the trunk and limbs (Wolpert et al.., 2002).

The ectoderm, the outermost germ layer of the post-gastrulation embryo, gives rise to the epidermis, neural plate and neural crest (Twyman, 2001; Wolpert et al.., 2002). The neural plate, which forms from the dorsal ectoderm, invaginates and fuses to form the neural tube and gives rise the central nervous system (Twyman, 2001; Wolpert et al.., 2002). The epidermis, which forms from the ventral and lateral ectoderm, forms the skin and cutaneous structures (e.g. feathers, hairs, claws etc.) (Twyman, 2001; Wolpert et al.., 2002). The neural crest produces most of the peripheral nervous system in addition to various other cell types (e.g. bone, connective tissue and smooth muscle) (Twyman, 2001; Wolpert et al.., 2002). By 9 days, gastrulation is complete and organogenesis commences and continues up until birth.

1.1.3 Zebrafish

In zebrafish, similar to the mouse, after fertilisation the zygote also undergoes cleavage. However, cleavage in zebrafish is restricted to the animal pole of the embryo resulting in a mound of blastomeres situated above the yolk (Kimmel et al.., 1995). Further cleavage results in a blastoderm, a single layer of flattened cells, overlying the yolk (Twyman, 2001; Wolpert et al.., 2002). After the mid-blastula transition the blastoderm becomes motile and begins to spread over the yolk by the process known as epiboly, the first major gastrulation cell movement. Gastrulation proper begins approximately at 50% epiboly (5.25 hours post fertilisation), which is the stage that the blastoderm has covered approximately half of the yolk (Kimmel et al.., 1995). Around 6 hpf a landmark event is reached known as the "shield stage" and represents the first clear morphological identification of the dorsal side of the embryo (Kimmel et al.., 1995). The zebrafish

"shield" is the gastrula dorsal organiser and is equivalent to the mouse "node". The shield tissue later differentiates into axial tissues, including the notochord (Saude et al.., 2000). Gastrulation is complete by 10 hours and the embryo has now reached the "tailbud" stage during which the embryo elongates and tissues begin to differentiate (Kimmel et al.., 1995).

Gastrulation is followed by neurulation and somitogenesis. Somites appear anteriorly at about 10.5 hpf and continue to develop sequentially in an anterior-posterior direction on either side of the notochord (Kimmel et al.., 1995). Concurrent with somitogenesis is the development of the nervous system. By 24 hpf somitogenesis is complete and the notochord is fully developed (Kimmel et al.., 1995). At 48 hours the embryos hatches and the young fish is able to swim (Kimmel et al.., 1995).

1.2 Central Nervous System

The vertebrate central nervous system (CNS), made up of the brain (forebrain, midbrain and hindbrain) and the spinal cord, executes the most complex functions of any organ system in the animal embryo. All the cells of the CNS derive from the neural plate, a flat sheet of epithelial cells, which towards the end of the gastrulation begins to fold to form the neural tube. Cells within the neural tube will give rise to the brain and the spinal cord. The neural tube progressively becomes regionalised along the anterior-posterior axis (A-P) and the dorsal-ventral axis (D-V) to give rise to distinct cell identities at discrete positions. These cells in turn will generate different types of mature neurons which will collectively create the vertebrate CNS.

1.2.1 Spinal Cord

Our research focuses on the patterning of the spinal cord. The relative simplicity of the spinal cord makes it a suitable system to study cell signalling and pattern formation and the generation of a well patterned spinal cord is the first step necessary for an animal to sense and respond to stimuli. Layers of neurons are generated along the dorsal-ventral axis of the spinal cord in a spatial and defined order. Broadly speaking, neurons generated in the dorsal neural tube respond to and process sensory input while neurons generated in the ventral neural tube coordinate motor output (Briscoe and Ericson, 2001). Spinal cord (SC) development proceeds in a bilaterally symmetric manner where different cell types are generated at different D-V positions. In the spinal cord future motor neurons are exclusively generated in ventral positions while sensory neurons derive dorsally on either side of the midline.

The generation of distinct neuronal subtypes arising from defined positions along the dorsal-ventral spinal cord axis has its origin in the inductive signals that emanate from organising centres (Caspary and Anderson, 2003; Poh et al.., 2002). Positional information derives from the ventral midline, the notochord, the dorsal midline and the somites. Two non-neuronal structures, the floor plate and the roof plate, develop at the ventral and dorsal midlines of the neural tube and have important roles in neuronal development and patterning acting as organising centres. Ventralising signals arise from the notochord and the floor plate while dorsalising signals are secreted from the dorsal epidermal ectoderm and the roof plate.

Signals from the surface ectoderm specify the roof plate (Dickinson et al.., 1995). Bone morphogenetic proteins (BMPs) that are expressed in the roof plate are believed to be involved with the induction of dorsal neural fates (Caspary and Anderson, 2003). Even though the role of BMPs in roof plate specification is not clarified in the mouse,

experiments on chick explants have shown that various BMPs can promote dorsal neural cell fates (Caspary and Anderson, 2003). In zebrafish, early BMP signalling is crucial for patterning the margin of the neural plate in regions rostral to the neural crest, while early dorsal markers have been shown to be spatially regulated by BMP activity (Barth et al.., 1999). It has also been proposed that Sonic Hedgehog (Shh) is required to restrict the expression of dorsal markers to their dorsal domains by opposing the activities of BMPs (Lee and Jessell, 1999).

Many other classes of secreted factors have also been shown to pattern the neural tube. These include fibroblast growth factors (FGFs), retinoids and Wnts. Retinoids, derived from the paraxial mesoderm, are known to be involved in the specification and differentiation of motor neurons and interneurons in the ventral and intermediate spinal cord (Pierani et al.., 1999). Exposure of prospective neural cells to FGFs, also secreted from the paraxial mesoderm, has been shown to impose a generic caudal neural character (Doniach, 1995; Storey et al.., 1998). Wnts, similar to BMPs, derive from the dorsal neural tube and they are believed to be involved in the patterning of the dorsal neural tube (Lee and Jessell, 1999).

Sonic Hedgehog (Shh) is also involved in the patterning of the neural tube. It has been characterised as a diffusible signal, secreted from the notochord and the floor plate, required for the correct patterning of the ventral neural tube (Roelink et al.., 1995). I will next consider floor plate induction and then discuss the Shh signalling and its role in ventral neural tube patterning.

1.3 Floor Plate Specification

In all vertebrates, floor plate cells occupy the ventral midline of the embryonic spinal cord. The floor plate plays important roles in the dorsoventral patterning and axonal guidance within the neural tube of vertebrate embryos (Dodd et al.., 1998). Both the notochord and the floor plate regulate ventral neural tube patterning in a similar way and they are both sources of the diffusible N-terminal peptide Shh signalling molecule (Placzek, 1995).

The importance of the floor plate as an organising centre has resulted in much attention being given to the mechanism of its induction. Several notochord transplantation and *ex vivo* experiments have shown that floor plate induction depends on signals from the notochord (Placzek et al.., 1991; Yamada et al.., 1991). The amino-terminal peptide of Shh replicates the floor plate inductive effects of notochord (Roelink et al.., 1995; Yamada et al.., 1993). Mis-expression of Shh was able to induce an ectopic floorplate *in vivo* (Roelink et al.., 1995). Additionally, elimination of Shh signalling from the notochord using mouse mutations or by antibody blockade *in vitro* prevents floor plate, motor neuron and ventral interneuron differentiation (Chiang et al.., 1996; Ericson et al.., 1996; Pierani et al.., 1999).

A critical event in floor plate development is the expression of a winged-helix/forkhead transcription factor FoxA2 (Ruiz i Altaba et al.., 1993). FoxA2 has been shown to be able to induce ectopic floor plate differentiation (Ruiz i Altaba et al.., 1995a; Ruiz i Altaba et al.., 1995b; Sasaki and Hogan, 1994). FoxA2 is a candidate target molecule of Shh signalling as its expression in the floor plate is induced by signals derived from the notochord (Chiang et al.., 1996; Ruiz i Altaba et al.., 1995a). Additionally, Shh is able to induce FoxA2 expression and it has been suggested that Gli proteins are involved in the

activation of FoxA2 as Gli binding sites are required for response to Shh signalling (Sasaki et al.., 1997).

Notochord ablation prevents floor plate cells from differentiating; the neural tube is smaller than normal and motorneurons fail to generate (reviewed in (Le Douarin and Halpern, 2000)). Studies on the chick embryo have led to a model where floor plate induction is mediated by notochord-derived Shh signals in a contact dependent manner (Placzek et al.., 1993).

However, this model of floor plate differentiation has been challenged and an alternative view has been proposed. According to this, the floor plate is not induced but it is generated through insertion, of a group of floor plate precursors, into the neural plate (reviewed in (Le Douarin and Halpern, 2000)). This is indicated by findings in zebrafish where Hedgehog signalling seems to play a less critical role in the floor plate specification (Odenthal et al.., 2000). In zebrafish, the floor plate consists of a single row of medial floor plate (MFP) cells flanked by what is known as lateral floor plate (LFP) cells.

Similar to other vertebrates, the zebrafish notochord is a source of Hh signalling expressing two of the hedgehog homologues found in the fish, Shh and echidna hedgehog (Ehh) (Currie and Ingham, 1996; Krauss et al.., 1993). MFP cells express Shh, tiggy-winkle hedgehog (Twhh), and the homologue of FoxA2, Axial1 (Ekker et al.., 1995; Krauss et al.., 1993; Strahle et al.., 1993). In contrast, LFP cells lack expression of any of the hedgehog genes but retain Axial1 expression (Odenthal et al.., 2000).

Fate mapping studies have indicated that the notochord and floor plate cells derive from a common precursor cell population, but during these studies no distinction was made between MFP and LFP cells (Amacher et al.., 2002; Shih and Fraser, 1995). Data have shown that Shh pathway is restricted to the formation of LFP cells while formation of MFP cells is initiated during early gastrulation and depends on one or more parallel pathways

involving the nodal related *Cyclops* (*cyc*) gene (Odenthal et al.., 2000). It has been suggested that MFP cells derive prior to in parallel with notochord formation while the LFP cells are induced at later stages (Odenthal et al.., 2000).These observations were supported by various studies and phenotypic analysis of zebrafish mutants with affected notochord and floor plate (MFP and FLP) cells (Odenthal et al.., 2000).

Although the contribution of different signals to floor plate induction in different species remains to be resolved, it appears that the role of the floor plate and Shh expression is conserved in inducing and patterning the vertebrate ventral neural tube (Jeong and Epstein, 2003). Shh signalling is responsible for the organising activity of the notochord and the floor plate within the vertebrate embryo. Failure to correctly initiate Shh expression in the notochord and the floor plate severely affects the development of ventral cell types in the spinal cord (Chiang et al.., 1996; Le Douarin and Halpern, 2000).

1.4 Sonic Hedgehog & Ventral Neural Tube Identity

1.4.1 Hedgehog (Hh) signalling pathway

Hh genes were discovered in 1980 by Nüsslein-Volhard and Wieschaus in a genetic screen for mutations that disrupted the *Drosophila* larval body plan. Several mutations identified caused the duplication of denticles, spiky cuticular processes, which decorate the anterior half of each body segment. Consequently, the appearance of a large number of denticles projecting from the larval cuticle resembled the spikes of a hedgehog and hence the name of one of these genes (Nusslein-Volhard and Wieschaus, 1980).

Numerous genetic studies on various fly mutants have provided most information on the Hh signalling pathway. More recently, cell culture work and vertebrate embryo studies suggest that the vertebrate Hh signalling pathway functions in a manner similar to

that in the fly. However, the molecular mechanisms of Hh signalling are best understood and analysed in *Drosophila.*

Hh proteins are synthesised as a ~45kD precursor that undergoes auto-proteolytic cleavage to generate a 25kD C terminal fragment with no identified function and a 19kD N-terminal fragment (Hh-N) known to be the one sufficient for all Hh signalling activity (Lee et al.., 1994). The Hh-N protein fragment undergoes further cholesterol and lipid modifications that even though their exact role remains unclear they have been thought to be involved in Hh protein sub-cellular localisation (Ingham, 2001; Ingham and McMahon, 2001).

It has been shown that a major function of the Hh gene in *Drosophila* embryos is the maintenance of the *wingless* (*wg*) gene, a member of the Wnt family of signalling molecules, at the boundary of each segmental unit (Hidalgo and Ingham, 1990; Ingham, 1993; Ingham and Hidalgo, 1993; Krauss et al.., 1993; Strahle et al.., 1993). Similarly three other segment polarity genes *smoothened* (*Smo*), *fused* (*Fu*) and *cubitus interruptus* (*Ci*) are also involved in the maintenance of expression of the wingless (*wg*) gene at the para-segmental borders (Ingham, 1998a; Ingham, 1998b). On the other hand the action of a fourth segment polarity gene *patched* (*Ptc*) was found to repress *wg* (Hidalgo and Ingham, 1990; Ingham and Hidalgo, 1993; Ingham et al.., 1991). By making double mutant combinations between the different segment polarity genes described it was established that *Smo*, *Fu* and *Ci* all act downstream of *Ptc* and result in *wg* transcriptional activation (Hooper, 1994).

Subsequent studies have confirmed Smo, Fu, Ci and Ptc as components of the Hh pathway. Biochemical reports indicated that activation of the Hh signalling pathway is initiated by stoichoimetric binding of the Hh ligand to the trans-membrane protein Ptc (Marigo et al.., 1996). In the absence of Hh, Ptc acts catalytically to suppress the activity of

Smo (Taipale et al.., 2002). Inactivation of Ptc, which is achieved by binding to Hh, allows activation of Smo, which in turn activates the transcription factor Ci a critical mediator of Hh signalling (Ingham, 1998a; Ingham, 1998b). Ci can exist in two forms: a repressing one (CiR) and an activating one. In the absence of Hh Ci is proteolytically processed into the truncated repressor form that results in inhibition of Hh target genes (Aza-Blanc et al.., 1997; Robbins et al.., 1997). In the presence of Hh the processing of Ci is inhibited and Ci is converted into a transcriptional activator (Methot and Basler, 1999; Ohlmeyer and Kalderon, 1998; Price and Kalderon, 2002).

1.4.2 Hedgehog (Hh) signalling pathway in vertebrates

Just over 10 years from their initial discovery in the fly, vertebrate Hh genes were reported (Echelard et al.., 1993; Krauss et al.., 1993; Riddle et al.., 1993). However, unlike the fruit fly that has a single Hh gene in vertebrate species there are a number of related genes present. There are three Hh genes that have been reported in the mouse namely *Desert hedgehog* (*Dhh*), *Indian hedgehog* (*Ihh*), and *Sonic hedgehog* (*Shh*) with *Dhh* being the one most closely related to the *Drosophila* Hh while *Ihh* and *Shh* were found to be closely related to each other (Echelard et al.., 1993). In zebrafish the Hh genes include *sonic hedgehog (shh)* and *tiggy-winkle hedgehog (twhh)* that are equally related to the mouse *Shh* and *echidna hedgehog (ehh)*, which is closely related to the mouse *Ihh* (Currie and Ingham, 1996; Ekker et al.., 1995; Krauss et al.., 1993).

The Hh signalling pathway has also been investigated in vertebrates where homologues of Ptc, Smo and Ci have been reported and it is analogous to a great extent with the Hh pathway in *Drosophila*. The three homologues of Hh in the mouse: *Dhh*, *Ihh* and *Shh* have distinct expression patterns and unique roles. Shh is involved in the dorsal-ventral patterning of the CNS, anterior-posterior patterning of the limb, the development of the somites, lungs and other organs. *Ihh* is involved in chondrocyte development and *Dhh* is required for the development of germ cells (Ingham, 2001; Ingham and McMahon, 2001). *Shh* is considered necessary for the correct patterning of the ventral nervous system since biochemical blockade of Shh signalling or genetic ablation results in the loss of ventral cell types in the vertebrate neural tube and cyclopia is observed (Chiang et al.., 1996).

There are two *Ptc* genes identified in mammals, *Ptc1* and *Ptc2*. Similar to *Drosophila*, in the absence of Hh, *Ptc1* prevents activation of the signalling pathway (Goodrich et al.., 1997). *Ptc2* binds all mammalian Hh proteins but it is still unclear

whether it has a role in the Hh signal transduction pathway (Carpenter et al.., 1998). Only a single *Smo* mammalian homologue has been reported to date (Akiyama et al.., 1997).

1.4.3 The Gli genes

Three vertebrate homologues to the *Drosophila Ci* have been identified: *Gli1*, *Gli2* and *Gli3* (Hui et al.., 1994; Marigo et al.., 1996). Their expression is partially overlapping in the neural tube and the evidence suggests they are the key transcriptional mediators of Shh signalling (Ingham, 2001; Ingham and McMahon, 2001; Lee et al.., 1997; Ruiz i Altaba, 1998). *Gli1* expression is restricted in the ventral half of the neural tube, its expression depends on Hh signalling and in contrast to *Gli2* and *Gli3* it only seems to function as activator of transcription (Hui et al.., 1994; Lee et al.., 1997; Ruiz i Altaba, 1998). *Gli2* expression is uniformly present in the neural tube while *Gli3* expression is restricted to the intermediate and dorsal neural tube (Lee et al.., 1997). Both Gli2 and Gli3 possess the N-terminal repressor domain as well as the C-terminal activator domain (Marigo et al.., 1996). However, the main activity of Gli2 seems to be as a transcriptional activator of Hh signalling contributing to the induction of most cell types in the neural tube while Gli3 mainly functions as a transcriptional repressor (Bai and Joyner, 2001; Bai et al.., 2004; Dai et al.., 1999; Ding et al.., 1998; Motoyama et al.., 1998; Persson et al.., 2002). A repressor form of Gli2 has been reported in the absence of Gli3 (Buttitta et al.., 2003). In zebrafish, Gli2 and not Gli1 was found to act as a transcriptional activator of the Hh pathway in the CNS while truncated Gli2 forms seemed to work as potent repressors (Karlstrom et al.., 2003).

Both mouse *Gli* knockouts and zebrafish *Gli* mutants have been reported. Disruption of *Gli1* in mice does not seem to cause any developmental defects and mice homozygous for *Gli1* are viable and appear normal (Bai et al.., 2002; Matise et al.., 1998;

Park et al.., 2000) suggesting that Gli1 does not act as a primary mediator of Shh signalling in development (Koebernick and Pieler, 2002). Mice mutant for *Gli2* have several affected tissues: neural tube, lungs, foregut, and skeleton (Akiyama et al.., 1997; Ding et al.., 1998; Mo et al.., 1997; Motoyama et al.., 1998). In the neural tube of *Gli2* mutants there is lack of floor plate and ventral interneurons (V3) but motor neurons (MNs) are still generated and they seem to expand ventrally suggesting that Gli2 is required to mediate Shh signalling in the ventral neural tube (Ding 1998, Jacob & Briscoe 2003). Finally, mice mutant for *Gli3* have severe defects in the forebrain and neural tube (Persson et al.., 2002; Theil et al.., 1999). The absence of *Gli3* causes a dorsal shift of the intermediate progenitor domains in the spinal cord and in some cases ectopic activation of Shh expression is observed (Persson et al.., 2002; Ruiz i Altaba, 1998). *Gli3* gene was found to be affected in human patients with Greig cephalopolysyndactyly syndrome (GCPS), an autosomal dominant disorder that affects the limbs and craniofacial development, and in the extra-toes^{J} (*Xt^{J}*) mouse, a spontaneous semi-dominant mouse mutation that affects limb development (Hui et al.., 1994).

Double mutations of *Gli* genes have also been reported in an attempt to understand in depth the role of *Gli* genes in mediating the Shh signalling pathway (Bai et al.., 2004; Ding et al.., 1998; Motoyama et al.., 1998; Park et al.., 2000; Persson et al.., 2002). In *Gli2;Gli3* double mutant mouse embryos, which lack all Gli function, motor neurons and V0-V2 interneurons are still generated but the different cell populations are extensively intermixed (Bai et al.., 2004). This suggested that Gli activity is not absolutely required for the generation of these neuronal types but is necessary for their correct positioning and number (Bai et al.., 2004).

In contrast to the mouse, *Gli1* zebrafish mutants, *Detour* (*dtr*), show a severe developmental phenotype (Karlstrom et al.., 1996; Karlstrom et al.., 2003). *Detour* mutants

lack lateral floor plate and show a reduced expression of Ptc1 (Karlstrom et al.., 2003; Odenthal et al.., 2000). Also in contrast to the mouse, in zebrafish embryos where Smo activity is lost weak Gli1 expression persists (Bai et al.., 2002; Karlstrom et al.., 2003). Studies of zebrafish *Gli1* have shown that Gli1 is necessary for patterning of the ventral CNS but it is not required for all Hh signalling since the development of somites, fins and dorsal aorta occur normally (Karlstrom et al.., 2003).

Gli2 zebrafish mutants, *you-too* (*yot*), have also been reported (Karlstrom et al.., 1999). *Yot* mutations encode carboxy-terminally truncated Gli2 proteins that retain the zinc finger DNA-binding domain but lack a region similar to a domain in *Drosophila* Ci implicated in binding dCBP, a transcriptional coactivator, as well as the VP16-like activation domain required for human Gli1 activity (Karlstrom et al.., 1999). Similar carboxy-terminal deletions impair the ability of Ci and Gli1 to activate Hh targets and share striking similarity to forms of Ci protein that transcriptionally repress Hh target genes and form by post-translational processing in the absence of Hh signalling (Aza-Blanc et al.., 1997; Karlstrom et al.., 1999). Moreover, *you-too* mutants have a reduced expression of Hh target genes, lack an optical chiasm and show developmental defects in the somites (Karlstrom et al.., 1999; Karlstrom et al.., 1996; Karlstrom et al.., 2003). These observations suggest that these mutant proteins are acting as repressors that form even in the presence of Hh and interfere with Hh signalling.

1.4.4 Shh signalling in the vertebrate ventral neural tube

Signals from the notochord provide positional information in the developing neural tube and act as the initial ventralising signalling source (Jessell, 2000). Shh appears to correspond to this signal. Previous studies have confirmed that Shh is both necessary and sufficient for the induction of ventral cell types in the spinal cord (Chiang et al.., 1996;

Ericson et al.., 1996). Shh is also required to restrict the expression of genes normally expressed in the dorsal neural tube, possibly by opposing the action of the bone morphogenetic proteins (BMPs) that control dorsal cell type specification in the neural tube (Lee and Jessell, 1999; Liem et al.., 2000)

In the ventral half of the neural tube five distinct progenitor domains give rise to five different neuronal subtypes. From ventral to dorsal the progenitor domains are p3, pMN, p2, p1 and p0. The neuronal subtypes derived from the p3, p2, p1 and p0 progenitors are V3, V2, V1, and V0 interneurons while pMN progenitors give rise to motorneurons (reviewed in (Briscoe and Ericson, 2001)). From ventral to dorsal, p3 progenitors express Nkx2.2 and Nkx6.1; pMN progenitors express Nkx6.1 and Pax6; p2 progenitors express Nkx6.1, Irx3, and Pax6; p1 express Dbx2, Irx3 and Pax6 and p0 progenitors express Dbx1, Dbx2, Irx3, and Pax6 (Briscoe and Ericson, 2001).

In vitro experiments established that all five classes of ventral neurons in the spinal cord are generated in response to different concentrations of Shh (Ericson et al.., 1996; Ericson et al.., 1997b). Moreover, the concentration threshold of Shh required to induce the neuronal subtypes *in vitro* corresponds to the position that these neurons are generated *in vivo*. Progressively higher Shh concentrations are required for the induction of neurons generated closer to the signalling source the notochord and floorplate (Figure 1).

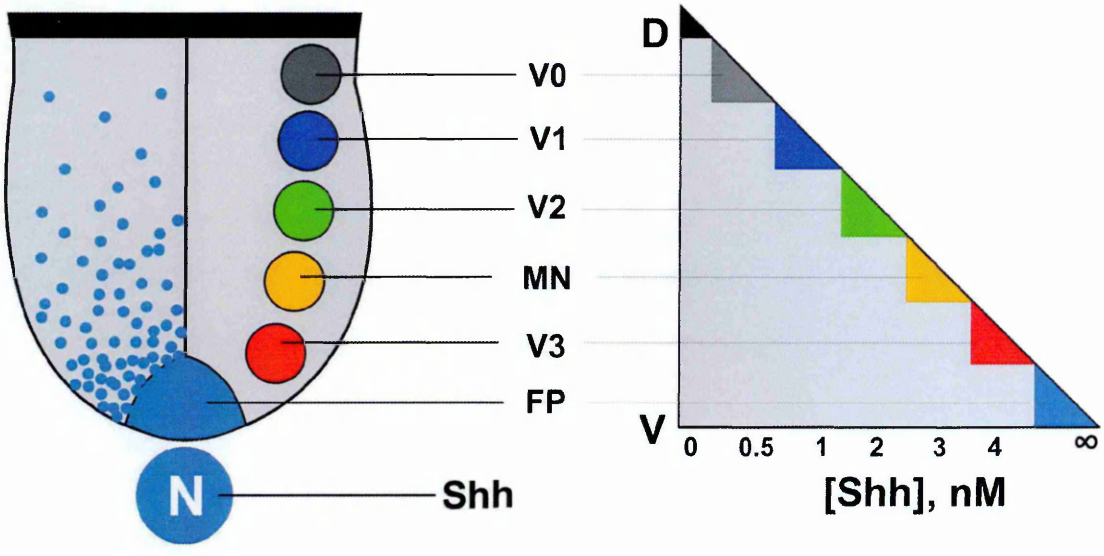

Figure 1. Shh is secreted from the notochord and the floor plate and induces different neuronal subtypes at different concentration thresholds (Briscoe and Ericson, 2001).

1.4.5 Interpretation of graded Shh signalling by neural progenitors

In vertebrates, Shh is produced by the notochord and the floor plate both structures known to be involved in the specification of the different cell identities in the neural tube (Echelard et al.., 1993; Krauss et al.., 1993). Shh has been proposed to act as a long range morphogen responsible for the patterning of the ventral neural tube (Briscoe et al.., 2001; Briscoe and Ericson, 2001). The term morphogen is used to describe a specific type of signalling molecule that derives from a source and spreads away from it to form a concentration gradient. This gradient will then in a dose dependent manner induce distinct cellular effects in the developing tissue. However, in order to correctly identify and analyse a morphogen one needs first to determine whether a signalling molecule meets certain criteria required to qualify it as a morphogen. These criteria include whether the signalling molecule is released from a localised source to form a long range concentration gradient such that cells at a distance from the source respond directly to the signal. Exposure of cells

to high or low concentrations of the morphogen should have distinct and predictable effects on gene expression and cell fate and patterning (Gurdon and Bourillot, 2001).

To explore the way different Shh concentrations induce distinct neuronal subtypes it is important to examine the way Shh regulates the expression of homeodomain (HD) proteins in ventral progenitor cells. According to work of Briscoe et al.. these HD proteins can be subdivided in two different classes based on whether they are positively or negatively regulated by Shh signalling (Briscoe et al.., 2000). Class I (Pax7, Pax6, Dbx1, Dbx2, and Irx3) proteins are repressed by Shh signalling while Class II (Nkx2.2, Nkx2.9, Nkx6.1, Nkx6.2 and Olig2) proteins are induced by Shh signalling at defined concentration thresholds (Briscoe et al.., 2000; Briscoe et al.., 1999; Novitch et al.., 2001). Cross-repressive interactions between the HD proteins expressed in adjacent domains define sharp boundaries of expression of Class I and Class II proteins (Briscoe and Ericson, 2001). The importance of the cross repressive interactions between complementary pairs of HD proteins in adjacent progenitor domains have been confirmed by gain- and loss-of-function studies (Briscoe et al.., 2000). Additionally, previously published data have shown that individual Class I and Class II proteins direct cell differentiation towards a specific neuronal fate and restrict the expression of genes responsible for establishing adjacent neuronal subtypes (Briscoe and Ericson, 2001; Briscoe et al.., 2000). The motor neuron inducing activity of Nkx6.1 (present in p2, p3 and pMN progenitor domains), for example, is restricted to the pMN domain by the presence of Nkx2.2 (in p3 domain) and Irx3 (in p2 domain) (Briscoe et al.., 2000).

1.4.6 Cyclopamine can inhibit Shh signalling

Cyclopamine is a teratogenic steroidal alkaloid purified from the lily species *Veratrum californicum* (Binns et al.., 1963). Administration of cyclopamine early in

development has been shown to block Shh signalling (by inhibiting Smo activity) and induces holoprosencephaly and cyclopia in chick and zebrafish embryos giving the same effect to that of the Shh mutations in mice and humans (Chiang et al.., 1996; Cooper et al.., 1998; Incardona et al.., 1998; Roessler et al.., 1996). Since Shh is not only involved in the patterning of the eyes and the brain but also in the patterning of the somites, an additional phenotypic observation in embryos treated with cyclopamine are abnormal somites; in cyclopamine treated zebrafish somites show a U-shape in contrast to the normal V-shape (Wolff et al.., 2003).

Exposure to ethanol, a cyclopamine solvent used in many studies, has also been shown to cause cyclopia in fish by preventing migration of the prechordal plate mesoderm to its correct position (Blader and Strahle, 1998).

1.5 Control of Cell Type Identity by Hh Signalling

1.5.1 Genetic analysis of Nkx2.2 and Pax6 mouse mutants

HD proteins Pax7, Pax3, Pax6, Dbx1, Dbx2 and Nkx2.2 are expressed by ventral progenitor cells and their expression is regulated by Shh signalling (Briscoe et al.., 1999; Ericson et al.., 1997b; Goulding et al.., 1993). These HD proteins are responsible for specifying the identity of each of the classes of post-mitotic neurons that will derive from the analogous progenitor domain. Studies in the chick have shown that misexpression of individual HD proteins can change the fate and the position in a predictable manner (Briscoe et al.., 2000). Moreover, predictable switches in progenitor domain identity and neuronal fate in mice in which individual class I and class II HD proteins have been inactivated by gene targeting have also been shown (Briscoe et al.., 1999; Ericson et al.., 1997b). Extensive studies on mice carrying mutations in Pax6 and Nkx2.2 genes have

provided us with a better understanding of how HD proteins regulate neuronal subtype identity and have provided us with a clearer view on Shh signalling.

In the ventral neural tube motor neurons and interneurons derive from the progenitor cell populations that express the HD proteins Pax6 or Nkx2.2 in response to graded Shh signalling (Briscoe et al.., 1999; Chiang et al.., 1996; Ericson et al.., 1997a; Ericson et al.., 1997b; Marti et al.., 1995). As mentioned earlier, somatic motor neurons derive from the pMN domain. The pMN domain shares a ventral boundary with the p3 domain, which generates the V3 interneurons, and a dorsal boundary with the p2 domain, which generates the V2 interneurons (Briscoe et al.., 2000; Ericson et al.., 1997b). The p2 progenitor domain shares a dorsal border with the p1 domain that generates V1 neurons (Briscoe et al.., 2000; Ericson et al.., 1997b). Mice lacking Nkx2.2 and Pax6 have been previously reported (Hill et al.., 1991; Sussel et al.., 1998). (Figure 2)

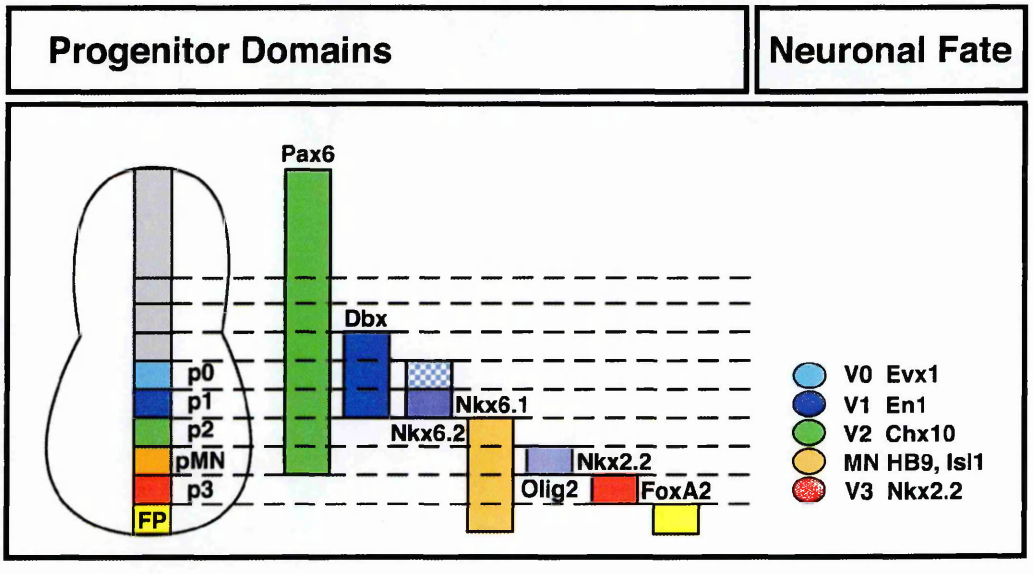

Figure2: The relationship between progenitor proteins and neuronal subtype identity

Nkx2.2 is expressed in the ventral-most domain of the neural tube located dorsolateral to FoxA2 expressing floor plate cells (Briscoe et al.., 1999; Ruiz i Altaba et al.., 1993). This ventral-most progenitor domain is also characterised by the expression of

another gene, Nkx2.9, a family member of the Nkx homeobox transcription factors (Briscoe et al.., 1999; Pabst et al.., 1998). Nkx2.9 expression in the spinal cord, in contrast to Nkx2.2, is almost extinguished after E10.5 while is still present at hindbrain levels (Briscoe et al.., 1999).

The analysis of *Nkx2.2* mutants has showed that Nkx2.2 is important in interpreting Shh signals and directing neuronal specification. In the absence of *Nkx2.2* the sharp boundary between Nkx2.2 (Class II protein) and Pax6 (Class I protein) remains unchanged (Briscoe et al.., 1999). Shh and FoxA2 expression are also not affected (Briscoe et al.., 1999). However, at spinal cord levels, in the absence of *Nkx2.2*, cells in the p3 domain no longer generate V3 interneurons but they instead generate motor neurons (MNs) (Briscoe et al.., 1999). V3 neurons express the basic helix-loop-helix (bHLH) gene *Ngn3* as well as *Sim1*, which contains both the bHLH domain and a PAS domain (Fan et al.., 1996; Sommer et al.., 1996). Expression of both genes (*Sim1* & *Ngn3*) is first detected when Nkx2.9 expression is almost extinguished in the spinal cord, around E10.5 (Briscoe et al.., 1999). In *Nkx2.2* mutants both Sim1 and Ngn3 are practically lost by E12.5 indicating that Nkx2.2 activity is necessary for V3 interneuron generation and it may also be needed for their maintenance (Briscoe et al.., 1999).

The generation of somatic motor neurons is defined by the expression of Isl1, Isl2 and HB9 (Briscoe et al.., 1999; Ericson et al.., 1997b; Tanabe et al.., 1998). In *Nkx2.2* mutants all three motor neuron markers are detected next to the floorplate, within the p3 progenitor domain (Briscoe et al.., 1999). The expression pattern of another homeodomain protein, Lim3 (progenitor and post-mitotic MN marker) (Tsuchida et al.., 1994), showed that MN generation ventrally in the Nkx2.2 mutants is due to their generation from the p3 domain and not due to migration from the pMN domain (Briscoe et al.., 1999). The ventrally generated motor neurons do not express Pax6 which indicates that Pax6 is not

directly needed for MN specification (Briscoe et al.., 1999). However, it has been suggested that Pax6 acts by repressing Nkx2.2 expression which in turn represses the generation of somatic motor neurons ventrally within the p3 domain (Briscoe et al.., 1999).

In contrast to the spinal cord, in the hindbrain instead of V3 neurons, visceral motor neurons are now generated from the ventral most progenitor domain p3 and express Phox2A/B (Briscoe et al.., 1999; Ericson et al.., 1997b). It was found that in the hindbrain, Nkx2.2 activity is not required for visceral motor neuron generation since in the absence of Nkx2.2 visceral motor neurons are generated normally (Briscoe et al.., 1999). Additionally there is no ventral expansion of motor neurons within the p3 domain (Briscoe et al.., 1999). A possible explanation for the hindbrain phenotype is the redundant activity of Nkx2.9 still present in the hindbrain of the Nkx2.2 mutants (Briscoe et al.., 1999). It has been suggested that the presence of Nkx2.9 in the hindbrain of *Nkx2.2* mutants is capable of rescuing the generation of V3 neurons by preventing the expansion of somatic motor neurons ventrally (Briscoe et al.., 1999).

Small Eye (Sey) mouse mutation has been suggested to be homologous to congenital aniridia in humans and *Pax6* was characterised to be identical to the mouse homologue of the candidate aniridia gene (Hill et al.., 1991). The phenotype of *Sey* mutant mice shows complete lack of eyes and nasal primordia (Hill et al.., 1991). Additionally lack of Pax6 causes incorrect patterning of the ventral spinal cord (Ericson et al.., 1997b).

In the absence of Pax6 the dorsal boundary of Nkx2.2 is no longer maintained and there is a dorsal expansion of Nkx2.2 within the region that would normally express Pax6 (Ericson et al.., 1997b) (Fig 29 & Fig 30). Further studies have shown that the Nkx2.2 expansion dorsally is not due to increased Shh signalling and that the domain populated by Nkx2.2 progenitor cells is defined indirectly through Shh mediated repression of Pax6 (Ericson et al.., 1997b).

In the mouse and the chick Pax6 progenitor cells generate V1 and V2 interneurons (Ericson et al.., 1997b) (Fig 29 & Fig 30). In homozygote *Sey* mice no V1 neurons were formed and V2 neurons were greatly reduced; reduced numbers of V2 neurons were observed in E11 and E12 *Sey/Sey* embryos (Ericson et al.., 1997b) (Fig 29 & Fig 30). Observations were based on the expression pattern of V1 interneuron markers such as En1 and Pax2 and V2 interneuron markers such as Chox10 and Lim3 (Ericson et al.., 1997b).

At spinal cord level, the dorsal Nkx2.2 expansion in the absence of Pax6 results in a reduction of somatic MN number accompanied by a dorsal expansion of Sim1 domain (Ericson et al.., 1997b) (Fig 29). In *Sey/Sey* embryos the conversion of progenitor cells from a motor neuron fate to a more ventral fate is characterised by the expression of Sim1. At hindbrain levels the total numbers of MNs does not change indicating that Pax6 is not directly responsible for MN generation (Ericson et al.., 1997b)(Fig 30). However, the absence of Pax6 results in a marked transformation of hypoglossal (h) MNs into visceral (v) MNs (Ericson et al.., 1997b).

1.5.2 Genetic analysis of Ngn3 mouse mutants

Ngn3 is a family member of a novel family of atonal-related basic-helix-loop-helix (bHLH) transcription factors (Sommer et al.., 1996). It is expressed in the ventral neural tube during neurogenesis and gliogenesis (Sommer et al.., 1996). bHLH transcription factors have been shown to be implicated in the determination and differentiation of glia. Members of the Olig family of bHLH transcription factors, Olig1 and Olig2, have been identified as regulators of oligodendrocyte specification (Lu et al.., 2000).

As mentioned previously, Ngn3 is both spatially and temporally expressed in the same ventral domain as Nkx2.2 and its expression is lost in Nkx2.2 mutants (Briscoe et al.., 1999). Similarly, in *Ngn3* mutants expression of Nkx2.2 and Sim1 (V3 interneuron marker)

was disrupted and virtually lost by E13.5 (Lee et al.., 2003). However, motor neuron generation, defined by isl1 expression, and V2 interneuron generation, defined by chox10 expression, appeared normal (Lee et al.., 2003). Therefore, Ngn3 appears to be involved in either the induction or maintenance of Nkx2.2 expression.

1.6 Neuronal Markers and Their Role in Zebrafish Development.

Despite the differences in the patterning during early embryonic development, vertebrates share various developmental mechanisms suggesting the presence of similar genetic programs. Over the last decade studies in zebrafish development has provided us with insights into a number of questions that were not clear by studies from other vertebrate model organisms. Zebrafish neural development has been extensively investigated. Stable transgenic lines and many mutations affecting neural development have been isolated and have provided detailed in vivo analysis of gene regulation (Lewis and Eisen, 2003; Udvadia and Linney, 2003).

The zebrafish spinal cord is patterned along the dorsal-ventral axis. Distinct cell types are positioned in specific dorsoventral domains; examples include floor plate cells and motorneurons that are located ventrally while neural crest derives dorsally. Various genes that control neuronal specification have been identified and their homology to other vertebrate genes has been confirmed. Additionally, similar to other vertebrates, hedgehog signalling is required for the specification of distinct neuronal subtypes including motorneurons and oligodendrocytes (Lewis and Eisen, 2003).

In zebrafish, in contrast to the mouse and the chick, shh is not the only hedgehog gene expressed in the notochord and the floorplate. Two more *Hh* genes, tiggy-winkle hedgehog (*twhh*) and echidna hedgehog (*ehh*) have been characterised (Currie and Ingham, 1996; Ekker et al.., 1995). The expression of Shh is seen in the floor plate and the notochord, Twhh is limited to the floor plate and Ehh is found exclusively in the notochord (Currie and Ingham, 1996; Ekker et al.., 1995). A well characterised floor plate marker commonly used in zebrafish studies is Axial1, which is the zebrafish homologue of the mouse FoxA2 (Strahle et al.., 1993).

Pax6 and *Nkx2.2* homologues have been reported in zebrafish (Barth and Wilson, 1995; Nornes et al.., 1998; Puschel et al.., 1992). Two *Pax6* homologues have been reported in zebrafish, namely *Pax6.1* and *Pax6.2* (Nornes et al.., 1998; Puschel et al.., 1992). In the spinal cord they show overlapping expression patterns and similarly to the mouse their expression is detected in the ventral half of the neural tube dorsally to the Nkx2.2 expression (Nornes et al.., 1998; Puschel et al.., 1992). The zebrafish Nkx2.2, homologue to the mouse Nkx2.2, is expressed in the ventral-most progenitor domain of the neural tube just above the floorplate resembling the mouse and chick expression (Barth and Wilson, 1995).

Zebrafish motorneurons and oligodendrocytes, similar to other vertebrates, derive form progenitor cells located in the ventral neural tube on either side of the floor plate (Lewis and Eisen, 2003; Park et al.., 2002). The zebrafish spinal cord has primary motorneurons that develop first and secondary motorneurons that develop later (Wolpert et al.., 2002). Fate mapping studies have shown that progenitor cells in the ventral most domain of the zebrafish neural tube are generating both primary and secondary MNs as well as oligodendrocytes (Kimmel et al.., 1994; Park et al.., 2002).

Studies of zebrafish mutants has shown that the number of primary motorneurons is proportional to the level of Hh signalling (reviewed in (Lewis and Eisen, 2003)). For example, in *cyclops* (*cyc*) mutants that lack floor plate and therefore shh and twhh signalling MNs appear normal (Hatta et al.., 1991); in *cyclops/floating* head (*cyc;flh*) double mutants that lack notochord and floor plate MNs are reduced severely (Beattie et al.., 1997); in *cyc;flh;syu* triple mutants that luck essentially all Hh signalling MNs are absent (Lewis and Eisen, 2001)Finally, morpholino knock down and mRNA over-expression studies have shown that *olig2*, a bHLH transcription factor, induces primary motor neuron differentiation and its expression is regulated by Hh signalling (Park et al.., 2002). However, the exact concentration and timing required for Hh signalling to pattern the ventral neural tube remains to be determined.

2. Materials and Methods

2.1 Embryo Manipulations

2.1.1 Zebrafish embryo collection

Zebrafish (*Danio rerio*) embryos were collected shortly after being laid and raised at 28°C in embryo water (red sea salt 0.03g/l, methylene blue 2mg/l) or in 0.3 Danieau's solution [1x Danieau solution consists of 58mM NaCl, 0.7mM KCl, 0.4mM $MgSO_4$, 0.6mM $Ca(NO_3)_2$, 5mM HEPES, pH 7.6]. Embryos were staged according to the morphological criteria provided in (Kimmel et al.., 1995).

Zebrafish embryos collected for *in situ* hybridisation were fixed in 4% PFA in PB [0.1M Phosphate Buffer (Table 3)] for a minimum period of 24 hours to a maximum of 72 hours (3 days) at 4°C. Following fixation, embryos were dehydrated in sequential washes with 100% methanol. Dehydrated embryos were stored in 100% methanol at -20°C until needed.

Zebrafish embryos collected for immunohistochemistry were fixed in 4% PFA in PB for 30 minutes (maximum 45 minutes) at room temperature. They were then washed in PBS and transferred to 30% Sucrose in PB for 1-2 hours. The embryos were mounted in O.C.T compound (BDH) and frozen on dry ice. Embryos were stored in -80°C until needed.

2.1.2 Mouse embryo collection

Embryos obtained from timed-pregnant matings were dissected from the uterus, placed in PBS and subsequently removed from their yolk sac and amnion. Embryos collected for staining procedures were transferred to 4% PFA in PB and left for 1 hour

(maximum 1.5 hours) at 4°C. They were then washed in PBS, placed in 30% Sucrose in PB and left overnight at 4°C. The next day the embryos were mounted in O.C.T compound (BDH) and subsequently frozen on dry ice. Embryos were stored in -80°C until needed.

2.1.3 Mouse and mouse embryo genotyping

Nkx2.2$^{+/-}$ mouse genotyping

Primers used (amplified fragment of Neo gene):

5'-AGAGGCTATTCGGCTATGACTG-3'

5'-CCTGATCGACAAGACCGGCTTC-3'

All PCR reactions were performed using an Eppendorf Thermal Cycler. The final concentration of the primers was 100ng per reaction. The final reaction volume was 40µl for which 4µl of DNA template was used. PCR Master-mix (AB-Gene) was used in all cases.

The PCR conditions were as follows:

94°C for 5min

29 cycles:

94°C for 1min

58°C for 2min

72°C for 3min

72°C for 10 min

The PCR products (400bp) were detected using a 2% agarose gel (Bio-Rad) in 1x TAE buffer.

Pax6$^{+/-}$ mouse genotyping

Primers used:

5'-GGGGGGTTTTCATCCTTTATG-3'

5'-CCGTTCCGATTTCTCTTATTGTC-3'

All PCR reactions were performed using an Eppendorf Thermal Cycler. The final concentration of the primers was 100ng per reaction. The final reaction volume was 40µl for which 4µl of DNA template was used. PCR Master-mix (AB-Gene) was used in all cases.

The PCR conditions were as follows:

94°C for 2min

29 cycles:

94°C for 30sec

55°C for 1min

72°C for 1min

72°C for 5.50min

The PCR products (16µl) were then digested using Dde I/Buffer 3 (BioLabs) for 2 hours at 37°C. The final products (~40bp) were then detected using a 4% metaphor gel (BMA) in 1x TAE buffer. Heterozygous mice could also be detected by examining the eye phenotype which varies from small eyes to no eyes at all.

Nkx2.2$^{-/-}$, Pax6$^{-/-}$ and Nkx2.2/Pax6$^{-/-}$ embryo genotyping

Genotyping of homozygote embryos was performed as described above or by immunohistochemistry for Nkx2.2 and Pax6 protein expression. Nkx2.2 was detected using rabbit anti-Nkx2.2 and Pax6 was detected with mouse anti-Pax6 antibody as described previously (Ericson et al.., 1997b).

Ngn3$^{+/-}$ mouse and Ngn3$^{-/-}$ embryo genotyping

Primers used (Wild type allele):

5'-CGGCAGATTTGAATGAGGGC-3'

5'-TCTCGCCTCTTCTGGCTTTC-3'

Primers used ($Ngn3^{\Delta}$ allele):

5'-CGGCAGATTTGAATGAGGGC-3'

5'-GCAGCGCATCGCCTTCTATC-3'

All PCR reactions were performed using an Eppendorf Thermal Cycler. The final concentration of the primers was 10μM per reaction. The final reaction volume was 20μl for which 0.5μl of DNA template was used. PCR Master-mix (AB-Gene) was used in all cases.

The PCR conditions were as follows:

94°C for 5min

35 cycles:

94°C for 30sec

60°C for 30sec

72°C for 40sec

72°C for 10min

The PCR products (~700 bp) were detected using a 2% agarose gel (Bio-Rad) in 1x TAE buffer.

2.1.4 Cyclopamine treatment

Cyclopamine (Toronto Research Chemicals Inc.) was dissolved at 10mM in 100% Ethanol and stored at -20°C. Embryos were incubated in various cyclopamine concentrations in 0.3x Danieau's solution, from different developmental stages until fixation, without chorion removal. In negative control experiments, the same quantity of 100% Ethanol was added to the 0.3x Danieau's solution.

2.1.5 Zebrafish whole-mount *in situ* hybridisation

Whole-mount *in situ* hybridisations were performed as described by (Thisse et al.., 1993) with a number of modifications. Zebrafish embryos were rehydrated in decreasing concentrations of methanol in PBT (Table 3) (75%, 50% and 25%) and then transferred in PBT (2 x 5min). The embryos were then re-fixed in 4% PFA in PB for 20 min. at room temperature and then washed in PBT. Embryos older than 24 hours were digested with proteinase K (10μm/ml) for 30 minutes and washed with PBT prior to fixation. After fixation embryos were transferred to hybridisation buffer (Hyb.) (50% formamide, 5xSSC (pH 7.0), 0.1% Tween-20, 50μg/ml heparin, 500μg/ml type VI torula RNA, 9mM citric acid to pH 6.0-6.5) for 2-5 hours at 68°C. The hybridisation buffer was then replaced with new hybridisation buffer containing 1μg/ml of DIG-labelled RNA probe and the embryos were incubated at 68°C overnight.

The following day washes performed at 68°C with preheated solutions 50% Hyb/2xSSC (5min), 100% 2xSSC (15min) and 100% 0.2xSSC (30min). A series of washes were performed at room temperature for 10 minutes each in 50% 0.2xSSC/PBT (twice) and 100% PBT. Embryos were blocked for several hours at room temperature in 2mg/ml BSA and 2% goat/sheep serum in PBT and then incubated overnight at 4°C with alkaline-phosphatase-conjugated anti-DIG antibody Fab fragments diluted 1:2500 in blocking buffer.

The day after, embryos were washed with PBT for a minimum of 8 times (15min). The embryos were then rinsed twice (5min) in NTMT (0.1MTris-HCl pH 9.5, 50mM $MgCl_2$, 0.1 M NaCl and 0.1% Tween 20). Detection was performed using NBT/BCIP (Roche ready made tablets/ 1 tablet dissolved in 10ml of distilled H_2O). The reaction was stopped with 2mM EDTA in PBS (pH 5.5) and embryos were re-fixed in 4% PFA in PB for 20 minutes at room temperature or overnight at 4°C. Embryos were then transferred to a

series of glycerol/PBT solutions (25%, 50%, 75% and 100%) and stored at 4°C in 100% glycerol. The yolk cell of the embryos was mechanically removed before photographing.

2.1.6 Mouse *in situ* hybridisation on cryosections

In situ hybridisations were performed as described by (Schaeren-Wiemers and Gerfin-Moser, 1993) with a number of modifications. Fresh or frozen slides were re-fixed in 4% PFA in PB for 10 minutes and then washed in PBS (3x 3min). The slides were acetylated for 10 minutes [4ml triethanolamine (Fluka) and 0.5ml concentrated HCl was added to 295ml H_2O and stirred; 0.75ml of acetic anhydride (Sigma) was added just before the sections were immersed] and again transferred to PBS (3x 5min).

Prehybridisation was performed at room temperature with 700µl hybridisation buffer [50% deionised formamide (Sigma), 5x SSC, 5x Denhardts, 10mg/ml herring sperm DNA (Promega), 10mg/ml bakers yeast RNA (Sigma)] per slide from a few hours to overnight in a humidified chamber. The Prehybridisation buffer was replaced with hybridisation mix (200ng of DIG-labelled RNA probe per ml hybridisation buffer). The hybridisation mix was heated for 5min at 80-100°C, to denature the probe, and then chilled on ice before added to the slides. The sections were then covered with coverslips (care was taken to avoid air bubbles) and placed in a humidified chamber (5x SSC/50% formamide) overnight at 70°C.

The next day the slides were washed by placing them vertically in a rack immersed in 5x SCC at room temperature and the coverslips were allowed to slide off. The slides were then washed in 0.2x SCC at 70°C for 1 hour and subsequently adjusted to room temperature in 0.2x SCC for 5min. The slides were transferred to buffer B1 (0.1M Tris.HCl pH 7.5, 0.15M NaCl) for 5min and then blocked for 1 hour at room temperature in buffer

B1 with 10% sheep serum. Anti-DIG antibody (dilution 1:5000) in buffer B1 with 1% sheep serum was added to the slides and left overnight at 4°C in a humidified chamber.

The slides were washed in buffer B1 (3x 5min) and then equilibrated in buffer B3 (0.1M Tris.HCl pH9.5, 0.1M NaCl, 50mM $MgCl_2$). Detection was performed using NBT/BCIP [4µl of 100mg/ml NBT in 70% dimethylformamide (Roche) and 4µl of 50mg/ml BCIP in 70% dimethylformamide (Roche) in 1ml of buffer B3]. 1µl of 2M levamisole was also added to the mixture before it was applied to the slides. The colour reaction was performed in the dark and stopped by transferring the slides in PBS (minimum 3 long washes). Sections were mounted in Vectashield (Vector) and stored at 4°C.

2.1.7 Immunohistochemistry on mouse and zebrafish cryosections

Blocking buffer (1%BSA / 0.1% Triton in PBS) was added to fresh or frozen sections for 5 minutes. The blocking buffer was then replaced with blocking buffer containing the primary antibody and left overnight at 4°C. The next day the slides were washed with PBS (4x 5min) and then the secondary antibody was added in blocking buffer. The slides were kept in the dark, at room temperature for 1.5-2 hours. The slides were washed with PBS (4x 5min). Vectashield (Vector), mounting medium for fluorescence and DAPI, was used for sealing the slides with coverslips. Primary antibodies used are listed in Table 1. Secondary antibodies FITC and Cy3 conjugated from Jackson Laboratories were used in the appropriate combinations.

2.1.8 Embryo photographing

High-power images (Normarski) from fixed embryos (whole and sections) were obtained using a Zeiss Axiophot microscope (Axioplan 2 imaging) fitted with an Axiocam HRC Zeiss digital camera and used Axiovision software. Samples subject to fluorescent

immunohistochemistry were imaged on a Leica confocal microscope (True confocal scanner Leica TCS SP II) with Leica confocal software. Images were processed with Adobe Photoshop.

2.2 General Molecular Biology Techniques

2.2.1 Small scale preparation of DNA

The Qiagen High Speed plasmid midi/maxi kit was (Qiagen) used for all small-scale plasmid preparations, according to the manufacturer's protocol.

2.2.2 DNA purification

DNA from rodent tails and yolk sacs was purified using the Qiagen DNeasy kit (Qiagen) according to manufacturer's specifications.

2.2.3 Nucleic acid quantification

Nucleic acid quantification was performed by spectrophotometry at λ = 260 nm, where an optical density (OD) unit corresponds to 50μg/ml of double-stranded DNA or to 40μg/ml single-stranded RNA. The ratio between the readings at λ= 260 nm and λ= 280 nm provided an estimate of the purity of the nucleic acid preparation (pure preparations of DNA should have OD_{260}/OD_{280} ratio of 1.8).

2.2.4 Gel electrophoresis

Nucleic acid size separation and size determination were performed by agarose gel electrophoresis. Gels were prepared by dissolving agarose (Bio-Rad) in 1x TAE [20mM TRIS acetate, 1mM $Na_2EDTA.2H_2O$ (pH 8.5)] to a final concentration of 0.8 – 2% (w/v), depending on the expected size of the DNA fragments, and 0.5 mg/ml ethidium bromide. Nucleic acid samples were mixed with 6x gel loading buffer (6x TAE, 50% v/v glycerol, and 0.25% w/v bromophenol blue). Electrophoresis was performed at 5 – 20 V/cm gel

length until appropriate resolution was achieved. Ethidium bromide-stained nucleic acid was visualised using ultraviolet light ($\lambda \approx 302$nm) and fragment size was estimated by comparison with the 1 kb ladder molecular weight markers (Invitrogen) run in at least one of the gel lanes.

For products smaller than 100bp a 4% metaphor gel (BMA) in 1x TAE buffer was used. The gel was made according to the manufacturer's protocol.

2.2.5 Phenol/chloroform extraction

To remove proteins from nucleic acid solutions, a mixture of phenol: chloroform: isoamyl-alcohol (25:24:1, volume ratio) was added in a 1:1 volume ratio to the nucleic acid solution and mixed for 1 minute. After a 5 min centrifugation (eppendorf centrifuge 5415D) at full speed (13,200 rpm), the upper (aqueous) layer was transferred into a new microcentrifuge tube and extracted again with an equal volume of chloroform: isoamyl-alcohol (24:1, volume ratio).

2.2.6 Ethanol precipitation of nucleic acids

Ethanol precipitation of nucleic acids was carried out by adding 1/10 volume of 3M sodium acetate (NaOAc, pH 5.5) and 3 volumes of 100% ethanol to the nucleic acid solution. This mixture was left at -20°C overnight. Centrifugation was carried out at 20,000 x g for 30min. The DNA pellet was washed in 70% ethanol and spun again at the same speed for 15 min. After ethanol removal nucleic acid was left to air-dry at room temperature (or at 37°C) for approximately 10 min and re-suspended in distilled H_2O.

2.2.7 Restriction digestion of DNA

Restriction enzyme digests were performed at the recommended temperature for approximately 2h, using commercially supplied restriction enzymes and buffers (Boehringer Mannheim, Promega, New England Biolabs). The enzyme component of the reaction never comprised more than 10% of the reaction volume.

2.2.8 Transformation of chemically competent bacteria

Transformation of the ligated vector or plasmid DNA was performed using chemically competent DH5-α *E. coli* cells that were made according to the protocol available at http://bioprotocol.bio.com/protocolstools/protocol.jhtml?id=p386. Up to 100ng of DNA was added to 100µl of cells thawed on ice. The bacterial cells were kept on ice for 15-30min and then heat shocked at 42°C for 1min followed by cooling in ice for a few minutes. 900µl of LB (Luria-Bertani Broth, 10g/l Tryptone, 5g/l yeast extract, 10g/l NaCl, pH 7.0) was added and the mixture was incubated at 37°C for 1 hour. An aliquot of 100µl from each transformation was spread onto a selective agar plate (0.1mg/ml ampicillin) and incubated overnight at 37°C.

2.2.9 Riboprobe synthesis

For the synthesis of riboprobes, plasmid DNA was linearised using the appropriate enzyme for 2 hours. DNA was extracted using phenol/chloroform extraction followed by ethanol precipitation. In vitro RNA transcription was performed at 37°C for 2 hours using in all cases digoxigenin (DIG)-labeled deoxy-uracil triphosphate (dUTP) (Roche). For a 20µl reaction we used 8.5µl of linearised plasmid, 2µl of 10x transcription buffer (Roche), 2µl 10x DIG-RNA labelling mix (Roche), 1.5µl RNase inhibitor (Roche), 4µl H_2O and 2µl RNA (T3, T7 or SP6) polymerase (Roche). Riboprobes were then treated with 20 U DNase

I (Roche) at 37°C for 15min to remove DNA template and were purified by size-exclusion chromatography through a DEPC water column (Clontech Chroma Spin-100). Using gel electrophoresis (1% agarose gel) the size and integrity of all riboprobes was checked prior to use. Riboprobes were added to hybridisation buffer shortly after synthesis and were stored at -20 °C. Table 2 has a list of all cDNAs used as templates for anti-sense RNA probes used in this work, as well as the respective origin.

Table 1 Primary antibodies used for immunohistochemistry

Epitope/Antigen	Species	Origin
FoxA2	Rabbit	(Ruiz i Altaba et al.., 1995a)
Shh	Mouse	(Ericson et al.., 1996)
Pax7	Mouse	(Ericson et al.., 1996)
Pax6	Mouse	(Ericson et al.., 1997b)
Phox2B	Rabbit	(Pattyn et al.., 1997)
Nkx2.2	Rabbit	(Ericson et al.., 1997b)
Olig2	Guinea pig	(Novitch et al.., 2001)
Evx1/2	Mouse	S. Morton
Chox10	Rabbit	(Liu et al.., 1994)
Gata3	Mouse	Santa Cruz
MNR2/HB9	Mouse	(Tanabe et al.., 1998)
Isl1/2	Mouse	(Ericson et al.., 1992)

Table 2 Templates for antisense RNA probes used in this thesis

cDNA	Linearisation	RNA polymerase	Origin
axial1	Sac I	T3	(Strahle et al.., 1993)
dbx1a	Sal I	SP6	(Campos, 2004)
ehh	Hind III	T7	(Currie and Ingham, 1996)
zevx	Sal I	SP6	(Campos, 2004)
gata2	Xba I	T7	(Detrich et al.., 1995)
zisl1	Xba I	T3	(Inoue et al.., 1994)
nkx2.2	BamH I	T7	(Barth and Wilson, 1995)
nkx6.1	Not I	SP6	(Campos, 2004)
nkx6.2	Not I	SP6	(Campos, 2004)
olig2	Not I	SP6	(Park et al.., 2002)
pax2.1	BamH I	T7	(Krauss et al.., 1991)
pax3	Not I	SP6	(Campos, 2004)
pax6.1	Not I	SP6	(Nornes et al.., 1998)
pax6.2	Not I	SP6	(Nornes et al.., 1998)
shh	Hind III	T7	(Krauss et al.., 1993)
twhh	BamH I	T7	(Ekker et al.., 1995)
Sim1	EcoR I	T7	(Fan et al.., 1996)
Ngn3	Hind III	SP6	(Sommer et al.., 1996)
FoxD3	BamH I	T3	(Kos et al.., 2001)
Nkx2.9	Sac I	T7	(Pabst et al.., 1998)
Pet1	Sac I	T7	(Fyodorov et al.., 1998)

Table 3 Formulation of frequently used solutions

Solution	Formulation
1x PBS	137mM NaCl, 2.7mM KCl, 4.3mM $Na_2HPO_4.7H_2O$, 1.4mM KH_2PO_4
1x PBT	1X PBS, 0.1% Tween-20
1x TAE	40mM Tris.Acetate, 2mM $Na_2EDTA.2H_2O$ (pH 8.5)
20x SSC	3M NaCl, 0.3M Na_2citrate.$2H_2O$, adjust pH to 7.0 with 1M HCl
Gel loading Buffer (6x)	6x TAE, 50% v/v glycerol, 0.25% w/v bromophenol blue
1M PB	0.6M $Na_2HPO_4.7H_2O$, 0.2M $NaH_2PO_4.H_2O$

3. Results

3.1 Dorsal-ventral patterning of the zebrafish spinal cord.

Many model organisms have been used by developmental biologists in efforts to understand the complex mechanisms that connect gene expression and cell fate specification with neuronal identity and behaviour. Zebrafish offer several advantages as a model organism including: low maintenance cost, rapid life cycle and easy collection of large numbers of transparent embryos. In addition to their transparent nature, zebrafish embryos develop entirely outside the mother allowing access at all stages of development. The established, stable lines of wild type and transgenic zebrafish along with translation-blocking morpholino injection technology make zebrafish a powerful tool for developmental studies.

The expression profiles of a series of genes from mouse and chick have been used extensively to address various developmental questions concerning the D-V patterning of the spinal cord. To establish whether zebrafish would provide a suitable model to study neural patterning of the spinal cord we first examined whether the markers used in mouse and chick are also expressed in a similar manner in zebrafish. We identified zebrafish homologues of these genes and determined their expression pattern. Information derived from these studies aimed to generate the reagents necessary for further studies of spinal cord patterning in zebrafish.

The markers were tested using *in situ* hybridisation and immunohistochemistry techniques. We focused our attention at 24 hours post fertilisation (hpf) because at this stage in zebrafish development somitogenesis is complete, the notochord is well developed

and the embryo possesses the classic vertebrate structure. At this stage of development we can effectively compare the CNS of the zebrafish to that of the mouse and the chick.

First, we looked at the expression of the three different zebrafish hedgehog genes, shh, ehh and twhh. Consistent with previous data (Krauss et al.., 1993) in 24hpf zebrafish embryos, *shh* expression in the neural tube is localised throughout the length of the floorplate (Fig 6). By 24hpf *shh* expression in the notochord is restricted caudally as well as to a group of undifferentiating cells in the expanding tail tip (Fig 6). However, at earlier developmental stages *shh* expression has been shown to be present throughout the length of the notochord (Krauss et al.., 1993). *Ehh* is expressed exclusively in the notochord at early developmental stages and gradually diminishes as somitogenesis progresses (Currie and Ingham, 1996). By 24hpf (30-somite stage) *ehh* expression is no longer detected in the notochord and expression is limited to a caudal region of the embryo (Fig 6). *Twhh*, 24hpf, is expressed strongly along the floorplate and unlike *shh*, *twhh* is not expressed in the notochord (Fig 6) (Ekker et al.., 1995).

We next examined a collection of markers that identify progenitor domains which will give rise to distinct neuronal subtypes in the vertebrate neural tube. Members of the Pax family proteins including Pax3, Pax6 and Pax7, are paired-homeodomain containing transcription factors. In both mouse and chick, Pax3 (Goulding et al.., 1991) and Pax7 (Jostes et al.., 1990) are expressed in dorsal neural tube progenitors, while Pax6 (Ericson et al.., 1997b; Goulding et al.., 1993) is expressed more broadly in both dorsal and ventral regions of the neural tube. As well as marking specific progenitor regions or neuronal subtypes these proteins have also been demonstrated to play important roles in neural patterning. (Ericson et al.., 1997b; Goulding et al.., 1993; Gruss and Walther, 1992).

Zebrafish homologues of the mammalian Pax3, Pax6 and Pax7 genes have been isolated previously (Puschel et al.., 1992; Seo et al.., 1998). The zebrafish contains two

Pax6 genes named Pax6.1 and Pax6.2 (Nornes et al.., 1998).We tested by *in situ* hybridisation *pax3*, *pax6.1* and *pax6.2*. Their expression patterns in the neural tube appeared to, in part, correlate with the expression patterns seen in the mouse and chick. Zebrafish *Pax3* was expressed throughout the anterior-posterior length of the embryo in the dorsal neural tube and was excluded from the ventral neural tube. This is similar to the expression observed in the mouse and the chick, but unlike the mouse and chick homologue zebrafish Pax3 was not detected in the dorsal-most part of the neural tube (Fig 3). *Pax6.1* and *Pax6.2* expression patterns also extended throughout the length of the dorsal neural tube (Fig 3). Moreover, their patterns of expression appeared to overlap and extend from the dorsal neural tube into intermediate regions (Fig 3) in a similar pattern to that observed in the mouse and the chick (Kawakami et al.., 1997; Walther and Gruss, 1991). Cross sections of zebrafish embryos showed that Pax3 expression domain was also overlapping with that of Pax6.1 and Pax6.2 dorsally, while no Pax3 expression was detectable in more ventral positions (Fig 3).

We also tested the mPax7 antibody on zebrafish cryosections. Similar to the expression of Pax7 in the mouse and chick spinal cord, zebrafish Pax7 expression was located in the dorsal part of the neural tube (Fig 7). Comparison of Pax7 expression pattern to that of *Pax3* suggest that Pax7 distribution is limited to a narrower expression domain close to the dorsal midline while Pax3 expression is detected in most of the alar plate (Fig 3 & Fig 7).

The Nkx2 and Nkx6 homeodomain proteins are also critically involved in the patterning of the neural tube. Studies in the spinal cord of the mouse and the chick have shown that Nkx2.2 is expressed in the ventral-most neuronal progenitor domain that generates V3 neurons and is responsible for suppressing motor neuron generation within that domain (Briscoe et al.., 1999). Like Nkx2.2, Nkx6.1 and Nkx6.2 are induced in the

ventral neural tube at early stages of neural development by Shh signalling but are expressed in a broader ventral domain compared with that of Nkx2.2. Genetic studies in mouse and chick indicate that they are required for the generation of somatic motorneurons (Cai et al.., 2001; Pattyn et al.., 2003; Qiu et al.., 1998).

We examined the expression of zebrafish homologues of *Nkx2.2* (Barth and Wilson, 1995), *Nkx6.1* and *Nkx6.2* (Isabel Campos, unpublished data) by *in situ* hybridisation in 24hpf wild type zebrafish embryos. We observed a correlation with the patterns of expression in the mouse and chick neural tube. *nkx2.2* is found in the ventral-most region of the neural tube (Fig 4) while *nkx6.1* and *nkx6.2* are expressed in a broader ventral neural tube domain with overlapping expression patterns (Fig 3). Additionally, using immunohistochemistry, we tested the mNkx2.2 antibody on zebrafish. These data indicate that the antibody recognised zebrafish Nkx2.2 and marked the ventral-most part of the neural tube adjacent to the floorplate region corresponding to the region where *nkx2.2* expression is localised (Fig 7).

Next, we examined Axial1, Olig2 and Dbx1a. Axial1 is the zebrafish homologue of FoxA2, a winged-helix transcription factor (Strahle et al.., 1993). FoxA2 expression is crucial for floor plate development and has been proposed as a possible direct target of Shh signalling (Sasaki and Hogan, 1994; Sasaki et al.., 1997). Axial1 has also shown to have an important role in the specification of the zebrafish ventral central nervous system (Strahle et al.., 1993). In the zebrafish, the floor plate consists of two distinct cell populations, the medial floor plate (MFP) cells and the lateral floor plate (LFP) cells (Odenthal et al.., 2000). Previous data have indicated that hedgehog signalling is only required for the induction of the LFP cells and not the MFP cells (Odenthal et al.., 2000). In 24hpf zebrafish embryos, Axial1 is expressed in the floorplate along the anterior-posterior axis of the spinal cord (Fig 4). Weak expression in the notochord has also been detected at earlier

developmental stages (8-10 somite stages) (Strahle et al.., 1993). The rabbit anti-FoxA2 antibody also marked the zebrafish floor plate domain providing an additional tool for identifying floor plate cells (Fig 7).

Olig genes, which encode basic helix-loop-helix transcription factors, are important for motor neuron and oligodendrocyte development (Novitch et al.., 2001). Studies from the mouse and the chick have shown that Olig2 is induced by Shh in the ventral neural tube and is expressed in the pMN progenitor domain (Lu et al.., 2000). Hedgehog signalling is also required for zebrafish Olig2 expression and oligodendrocyte development (Park et al.., 2002). Using *in situ* RNA hybridisation in 24hpf zebrafish embryos we detected *Olig2* expression in the ventral neural tube along the anterior-posterior neural tube (Fig 4). Transverse, zebrafish spinal cord, sections confirmed the expression of Olig2 in the pMN domain, dorsally to Nkx2.2, where mature motor neurons will derive (Fig 4).

Class I genes Dbx1 and Dbx2 are markers of the intermediate neural tube that includes the progenitors of V0 and V1 interneurons (Pierani et al.., 1999; Pierani et al.., 2001). Low levels of Shh signalling are required for the induction of Dbx1 and Dbx2 (Briscoe et al.., 2001) while high levels of Shh signalling prevent Dbx protein expression (Pierani et al.., 1999). In zebrafish three Dbx (Hlx) genes have been isolated (Seo et al.., 1999). Dbx1a and Dbx1b (hlx1 and hlx2) have an approximate 60% sequence homology to the mouse Dbx1 suggesting that they derive from a duplication in the fish lineage (Seo et al.., 1999). In zebrafish embryos, 24hpf, Dbx1a is expressed in the intermediate neural tube in a similar domain to that of the Dbx1 in the mouse (Fig 4).

We next turned our attention to neuronal markers. The expression patterns of Pax2.1, Islet1, Gata2 and Evx were examined. The zebrafish contains two Pax2 genes named Pax2.1 and Pax2.2 (Pfeffer et al.., 1998). However, Pax2.1 gene is most closely related to the mammalian Pax2 gene in its expression pattern as it is expressed first, prior to

somitogenesis, followed by Pax2.2 at 5 somite stage (Pfeffer et al.., 1998). Moreover, Pax2.1 in contrast to Pax2.2 is expressed in the nephric system (pronephros and nephric ducts) (Pfeffer et al.., 1998). Whole mount *in situ* hybridisation of zebrafish embryos showed that *pax2.1* is expressed in the intermediate and dorsal part of the neural tube 24hpf (Fig 5). Cross sections showed lateral positioning of *pax2.1* consistent with these cells representing newly generated post mitotic neurons (Fig 5).

Somatic motor neurons in the embryonic spinal cord express Islet1, a LIM homeodomain transcription factor (Ericson et al.., 1992) and the homeodomain protein HB9 (Harrison et al.., 1994). Both Islet1 (Isl1) and HB9 expression are required for the generation of motor neurons (Pfaff et al.., 1996; Tanabe et al.., 1998). The zebrafish homologue of Islet1 has been isolated previously (Inoue et al.., 1994) and in zebrafish embryos, 24hpf, the expression pattern is observed in the ventral neural tube in the same region that motorneurons derive (Fig 5). Cross sections from 24hpf zebrafish embryos showed Islet1 expression restricted to a group of cells laterally (Fig 5). HB9 and islet1/2 antibodies that mark motorneuron populations in the developing spinal cord of the mouse and the chick were tested on zebrafish cryosections. HB9 was shown to also mark motorneurons in the spinal cord of zebrafish (Fig 7). HB9 positive cells in either side of the ventral neural tube in 24hpf zebrafish cryosections were detected (Fig 7). In contrast to HB9, guinea pig Islet1/2 antibody only marked Rohon Bead neurons (dorsally) and not MNs, as seen in cryosections of 24hpf zebrafish embryos (Fig 7). Previous published work using rat Islet1 antibody indicated that this antibody was able to recognise Islet expression in both subtypes of neurons in zebrafish at 10hpf (Korzh et al.., 1993) and this expression is maintained until later on in somitogenesis (Korzh et al.., 1993). In contrast, our results with guinea pig Islet1/2 antibody indicate that this antibody does not efficiently recognise Islet

expression in MNs. This observation may reflect different affinity for Islet 1 and Islet 2 proteins or possible post-translational modification of Islet.

Gata2, a zinc-finger transcription factor, is a V2 neuronal marker and is required for the generation of V2b interneurons (Ericson et al.., 1997b; Zhou et al.., 2000). Evx, a homeodomain transcription factor, is a V0 neuronal marker whose expression is required for the differentiation of V0 interneurons (Moran-Rivard et al.., 2001). In zebrafish (24hpf) the Gata2 homologue (Detrich et al.., 1995) is expressed in the ventral neural tube in the domain from which interneurons will derive (Fig 5) while cells that express zebrafish Evx (Thaeron et al.., 2000) are located dorsal to the Gata2 positive cells (Fig 5). This was also shown by using the mEvx antibody on zebrafish cryosections (Fig 7). Both Gata2 and Evx seem to represent similar cell populations to that seen in the mouse and the chick.

We also examined the expression patterns of msxB and msxC, zash1 and zash5 and xash3 genes but no expression was detected in the neural tube in 24hpf zebrafish embryos. The zebrafish msxB and msxC are members of the msx homeobox genes and closely related to mouse msx3 which is expressed in the dorsal neural tube (Ekker et al.., 1997; Shimeld et al.., 1996). Zash1 and zash5 are two achaete-scute homologue (ash) genes and with zash1 closely related to rat Mash1 (Allende and Weinberg, 1994). Xenopus xash3 is a basic helix-loop-helix-containing gene and is expressed during early neurogenesis in the CNS (Turner and Weintraub, 1994).

The different DV markers described in this chapter show similar spinal cord expression patterns as in the mouse and the chick and provide a set of reagents that can be used for further developmental studies.

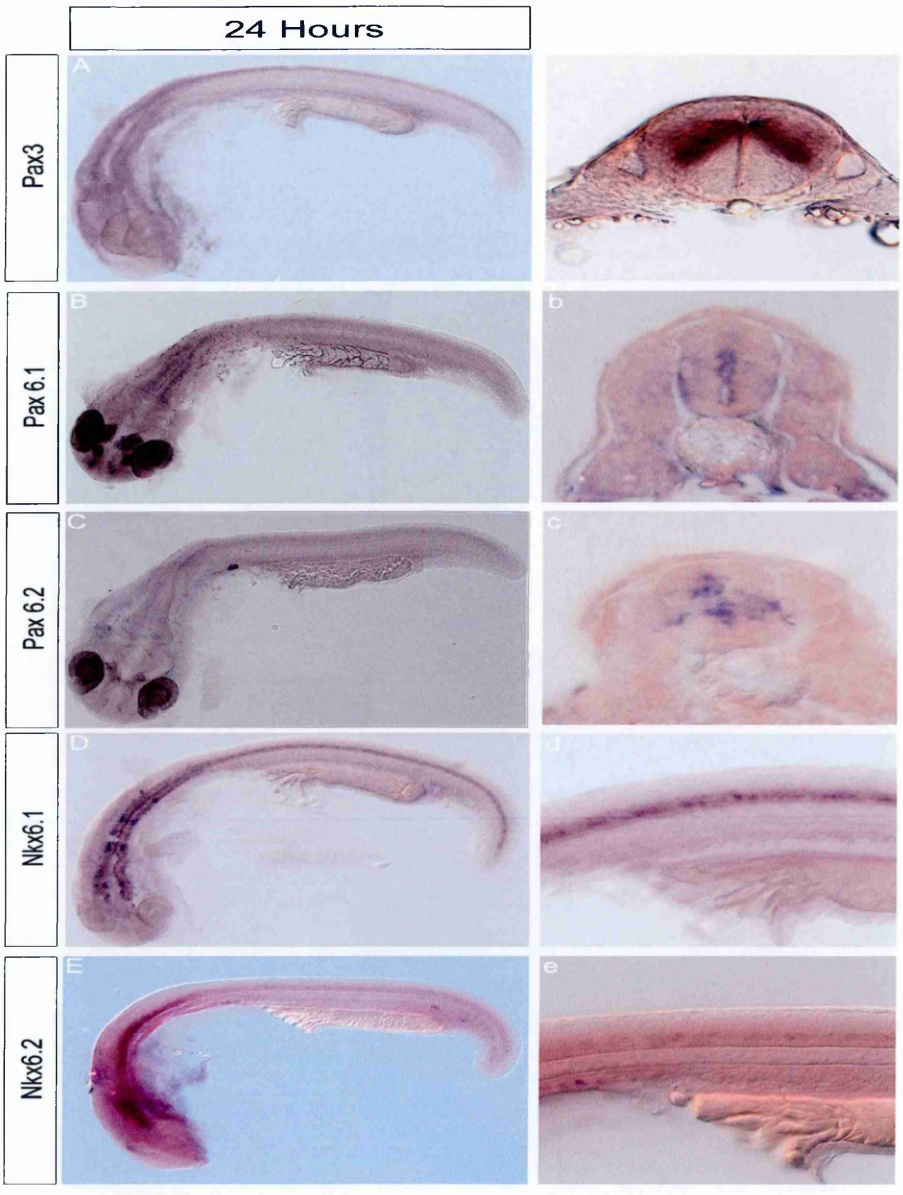

Figure 3: Expression pattern of progenitor markers in zebrafish spinal cord (A).

(A-E) Expression of progenitor markers in zebrafish 24hpf. (*In situ* hybridisation) [Anterior (left) - Posterior (right)] *Pax3* is expressed in the dorsal neural tube (A), *Pax6.1* (B) & *Pax6.2* (C) are expressed in the dorsal and intermediate neural tube and *Nkx6.1* (D) & *Nkx6.2* (E) expression is located in the ventral neural tube.

(a-c) Transverse sections through the zebrafish spinal cord showing the expression of *Pax3* (caudal hindbrain region) (a), *Pax6.1* (b) and *Pax6.2* (c) [Doral (top) – Ventral (bottom)]

(d-e) Higher magnification photos of the zebrafish tail showing the expression of *Nkx6.1* (d) and *Nkx6.2* (e) [Anterior (left) - Posterior (right)].

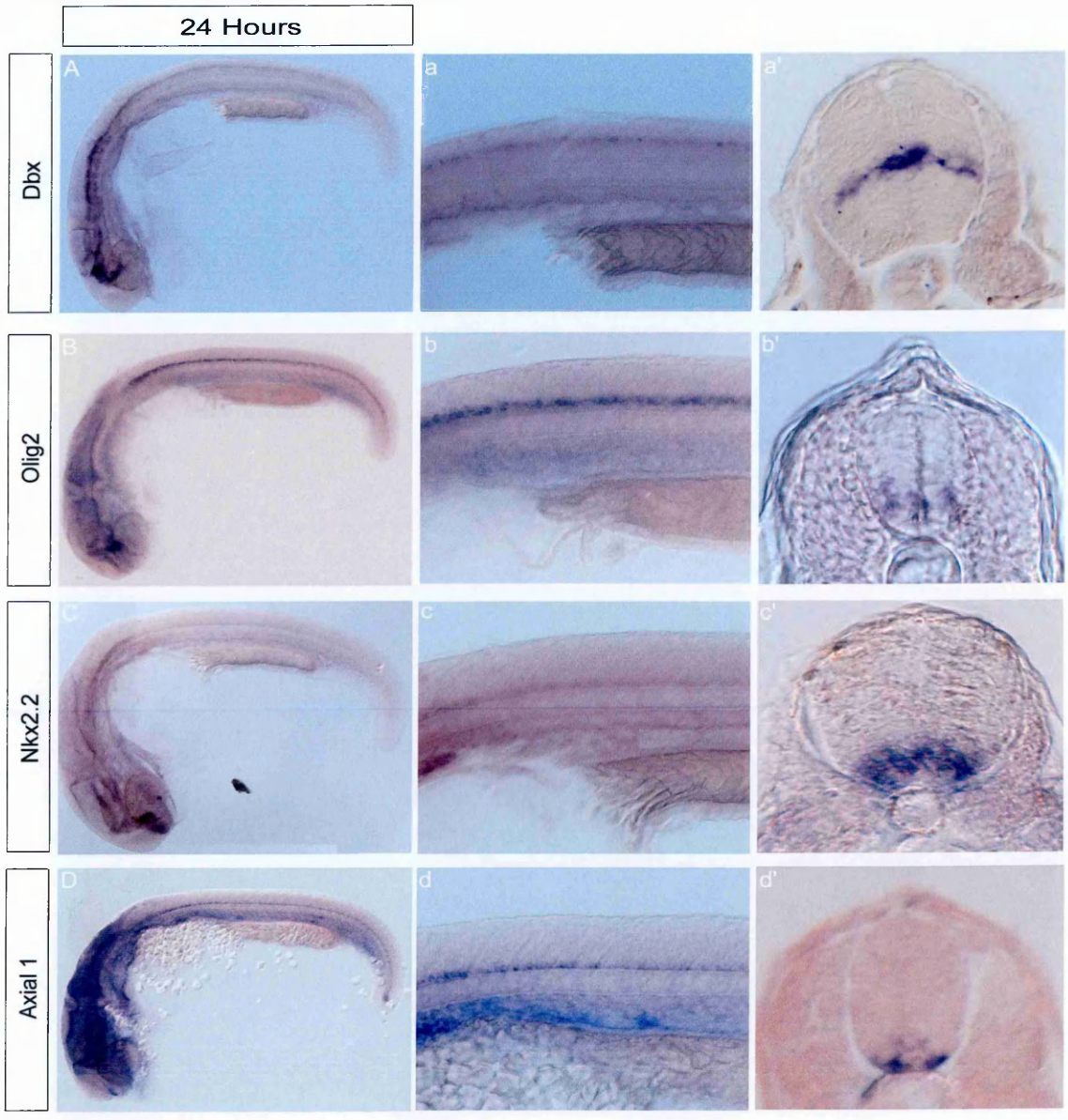

Figure 4: Expression pattern of progenitor markers in zebrafish spinal cord (B).

(A-D) Expression of progenitor markers in zebrafish 24hpf. (*In situ* hybridisation) [Anterior (left) - Posterior (right)] *Dbx* (A) is expressed in the intermediate neural tube, *Olig2* (B) is expressed in the ventral neural tube, *Nkx2.2* (C) expression is located in the ventral neural tube and *Axial1*(D) is a floor plate marker.

(a-d) Higher magnification photos of the zebrafish tail showing the expression of *Dbx* (a), *Olig2* (b), *Nkx2.2* (c) and *Axial1* (d) [Anterior (left) - Posterior (right)].

(a'-d') Transverse sections through the zebrafish spinal cord showing the expression of *Dbx* (a), *Olig2* (b), *Nkx2.2* (c) and *Axial1* (d) [Doral (top) – Ventral (bottom)].

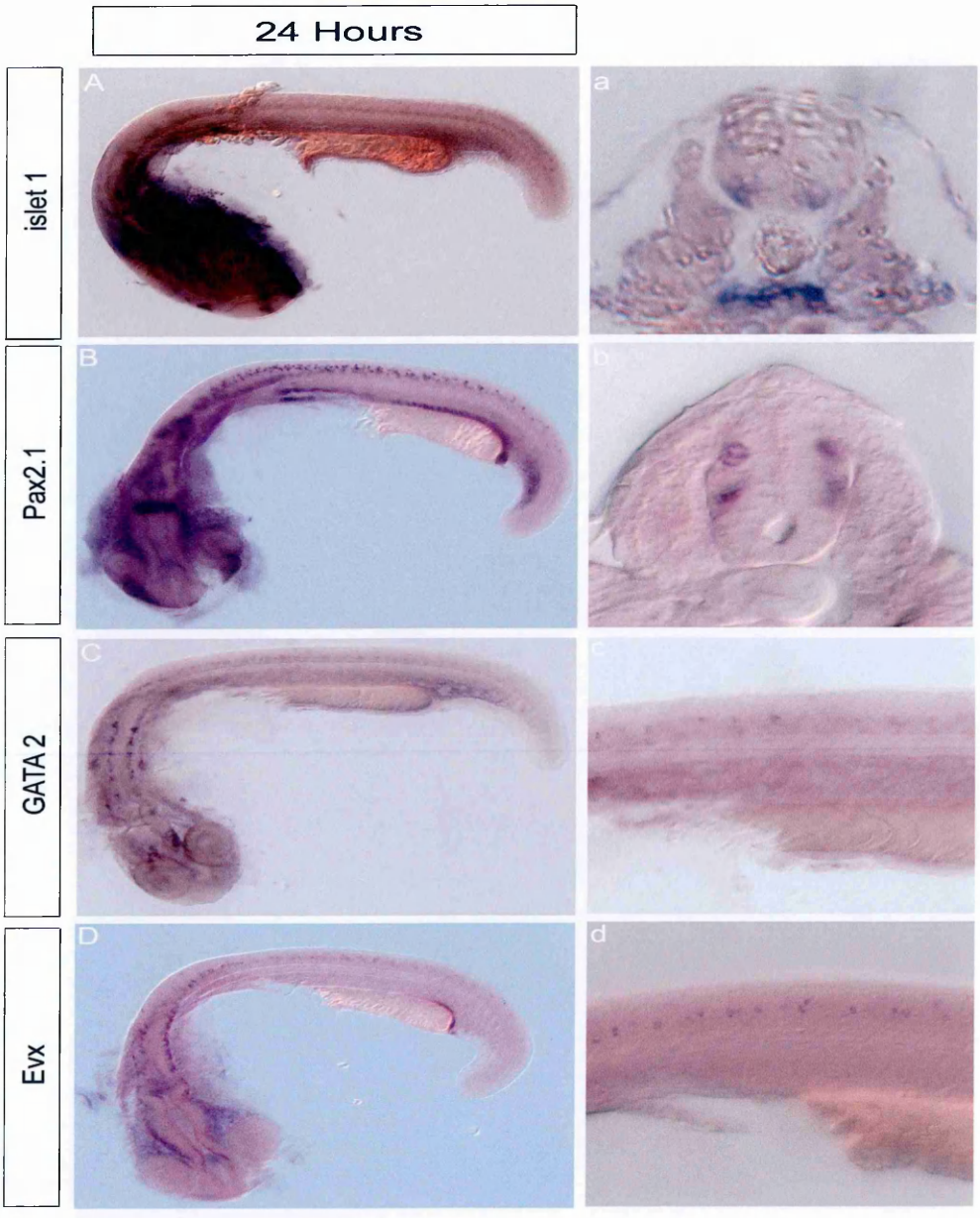

Figure 5: Expression pattern of neuronal markers in zebrafish spinal cord.

(A-D) Expression of neuronal markers in zebrafish 24hpf. (I*n situ* hybridisation) [Anterior (left) - Posterior (right)] *Islet1* (A) is expressed in the ventral neural tube, *Pax2.1* (B) is expressed in the intermediate and dorsal neural tube, *GATA2* (C) and *Evx* (D) expression is located in the ventral neural tube.

(a-b) Transverse sections through the zebrafish spinal cord showing the expression of *Islet1* (a) and *Pax2.1* (b) [Doral (top) – Ventral (bottom)].

(c-d) Higher magnification photos of the zebrafish tail showing the expression of *GATA2* (a), *Evx* (b) [Anterior (left) - Posterior (right)].

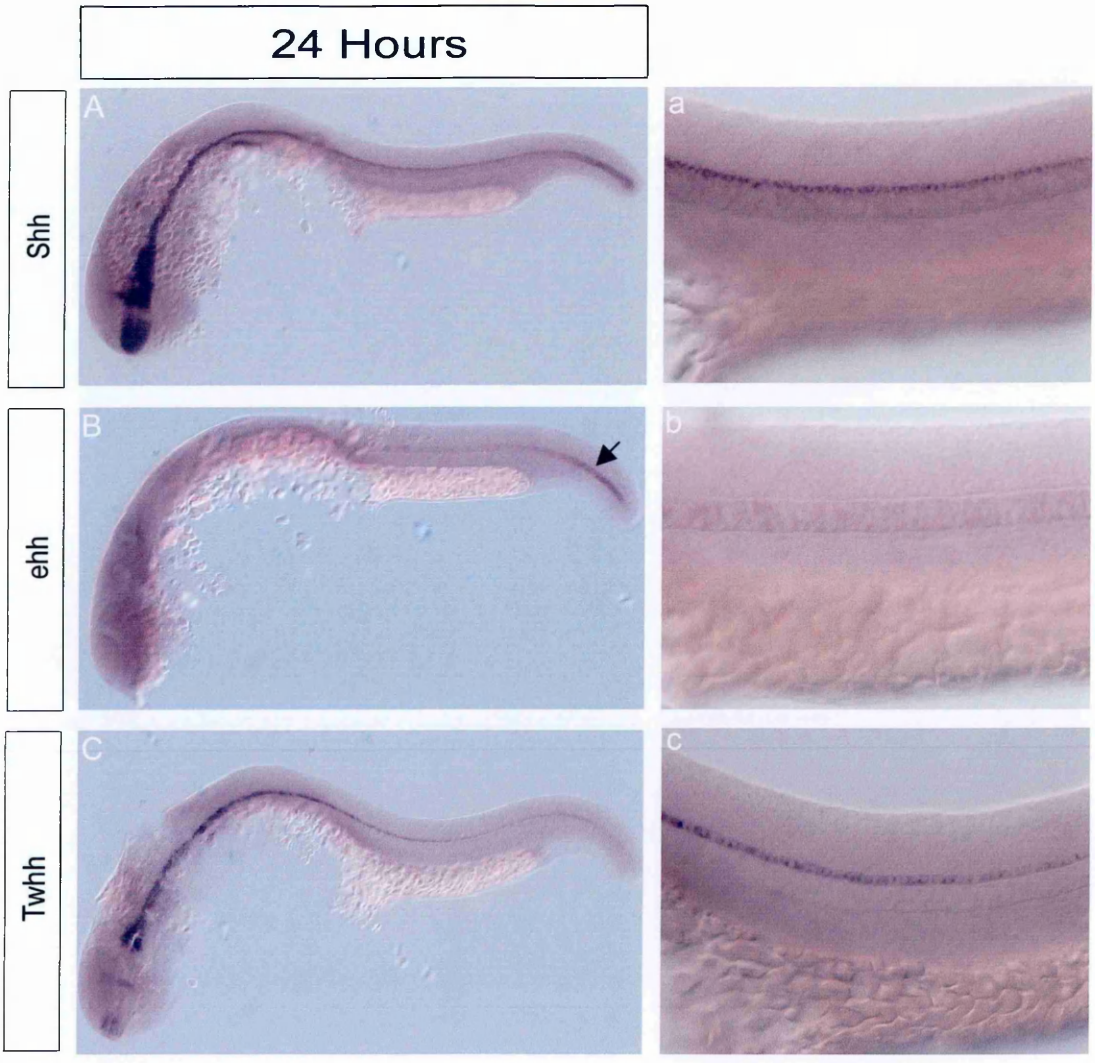

Figure 6: Expression pattern of the three hedgehog genes in zebrafish spinal cord.

(A-C) Expression of *shh, ehh* and *twhh* in zebrafish 24hpf. (I*n situ* hybridisation) [Anterior (left) - Posterior (right)] *Shh* is expressed in the notochord and the floor plate (A), *twhh* (C) is expressed in the floor plate while *ehh* (B) expression is limited in the notochord caudally (arrow).

(a-c) Higher magnification photos of the zebrafish tail showing the expression of *shh* (a), *ehh* (b) and *twhh* (c) [Anterior (left) - Posterior (right)].

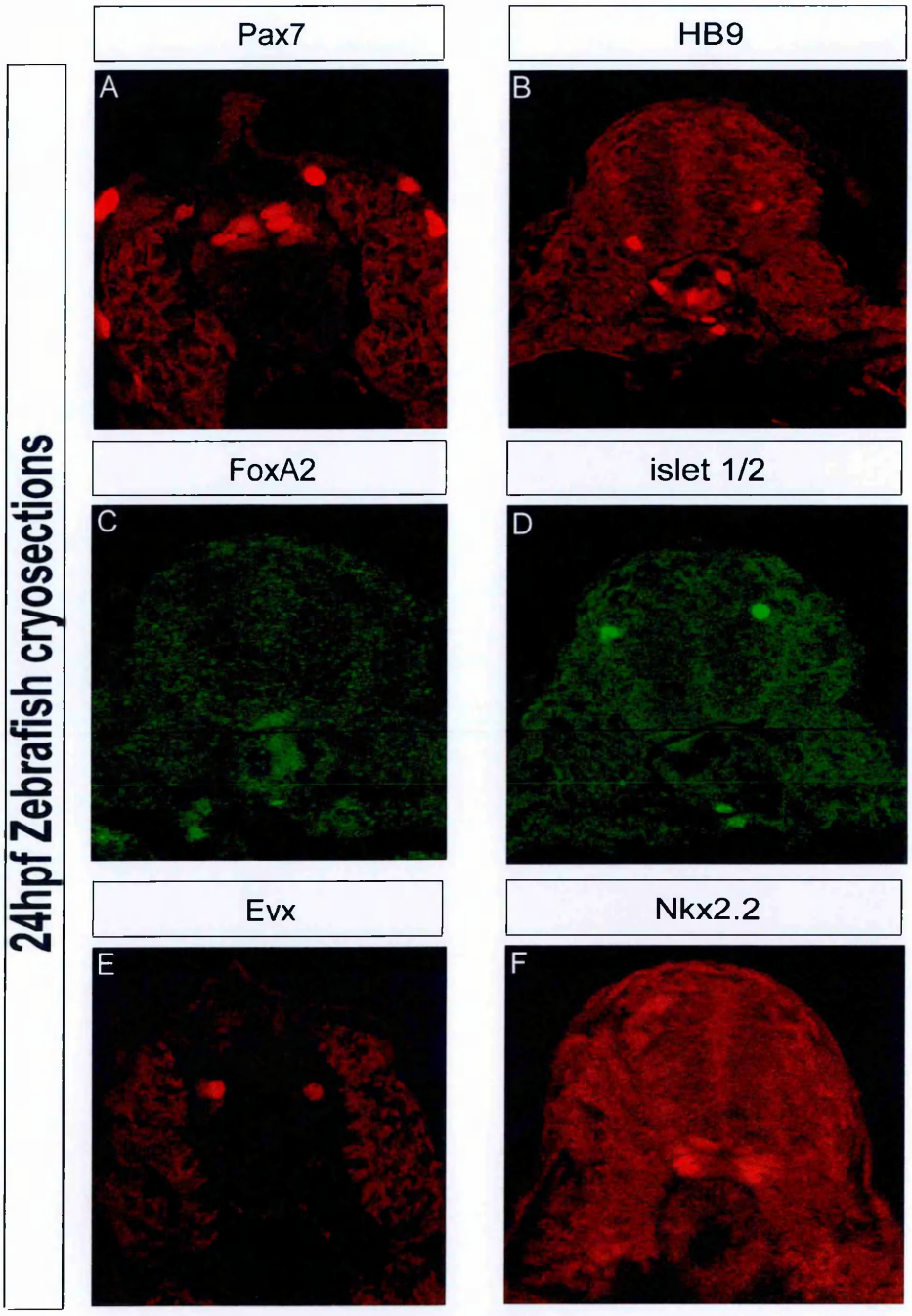

Figure 7: Antibody staining of zebrafish cryosections 24hpf.

[Doral (top) – Ventral (bottom)] (A) Mouse anti-Pax7 antibody staining Pax7 expressing cells in the dorsal neural tube (B) HB9 can be used as a motorneuron marker (C) Rabbit anti-FoxA2 antibody marked successfully the floor plate region (D) guinea pig islet1 1/2 antibody marked dorsal Rohon Bead cells but not ventral MNs (E) mouse anti-Evx antibody marked interneurons in the dorsal neural tube and (F) mouse anti-Nkx2.2 antibody marked the ventral most region of the zebrafish neural tube.

4. Results

4.1 Temporal and concentration requirements for Hedgehog (Hh) signalling during zebrafish spinal cord development.

Once we had established the normal pattern of expression of D-V genes in 24 hpf zebrafish we turned our attention to Hedgehog (Hh) signalling. Our aim was to characterize the concentration and temporal requirements of Hedgehog signalling for zebrafish neural tube patterning. The questions we wanted to address were the following:

1. Does blockade of Hh signalling inhibit ventral neural patterning?

   If so:

2. What are the temporal requirements for Hh signalling?
3. Is there a concentration dependence requirement for Hh signalling?
4. Is there evidence that different genes are differentially sensitive to Hh signalling?

To test whether Hh signalling is necessary for patterning the zebrafish ventral neural tube we took advantage of cyclopamine, which is a teratogenic steroidal alkaloid. Cyclopamine has been previously shown to work by blocking Hh signalling in chick and zebrafish embryos and give the same effect as null mutants of Shh in the mice (Chiang et al.., 1996; Cooper et al.., 1998; Incardona et al.., 1998).

Previous data from zebrafish experiments suggested that 25μM of cyclopamine concentration was sufficient to completely block Hh signalling (Campos, 2004). To test this we incubated wild type zebrafish embryos in 25μM of cyclopamine from 1 cell stage (0.2hpf) to 24 hours at 28°C. We then analysed the expression pattern of various molecular markers by *in situ* hybridisation and compared their expression pattern with that seen in the

mouse and the chick when Shh signalling is lost. We predicted loss of ventral neural tube markers, such as Olig2 and Nkx2.2, since their induction appears to depend on Hh signalling and ventral expansion of intermediate and dorsal molecular markers, such as Dbx and Pax3, since these are thought to be repressed by Hh signalling.

Wild type embryos left to grow normally at 28°C for 24 hours were used as the comparison group. Since ethanol was used as the solvent in which cyclopamine was dissolved we wanted to exclude any effects on the neural tube patterning due to the presence of ethanol. We therefore incubated wild type embryos from 1 cell stage to 24 hours in 0.1% of ethanol solution. Ethanol did not interfere with the ventral or dorsal expression of neural markers (Fig 8 & Fig 9). However, all the ethanol treated embryos demonstrated cyclopia. This is consistent with previously published data that have shown that exposure to ethanol causes cyclopia in fish by preventing migration of the prechordal plate mesoderm to its correct position (Blader and Strahle, 1998).

Having established that ethanol does not affect D-V patterning of the neural tube we first examined the effect of cyclopamine on the ventral neural tube markers *Olig2* (motor neuron marker) and *Nkx2.2* (ventral-most region of the neural tube). The expression of both markers was lost (Fig 9) when embryos were incubated in 25μM cyclopamine indicating that 25μM of cyclopamine concentration was sufficient to block Hh signalling. This also suggests that Hh signalling is required for the correct patterning of the ventral neural tube and the induction of different ventral neuronal subtypes.

We next examined the effect of cyclopamine on the floorplate marker, *Axial1*. In the mouse and the chick, the absence of Hh signalling blocks floor plate induction, therefore we would expect loss of *Axial1* in zebrafish treated with cyclopamine. However, *Axial1* was not consistently lost in embryos grown in 25μM cyclopamine (Fig 9 & data not shown). In most cases some decrease of *Axial1* was observed but not complete loss. This

may be explained by the evidence that in the fish, Hh signalling is required only to induce lateral floorplate cells and not medial floorplate cells (Odenthal et al.., 2000). Medial floorplate cells are unaffected by loss of Hh signalling and it has been suggested that they originate in parallel to notochord formation (Odenthal et al.., 2000).

We next turned our attention to *Dbx1a*. This gene is expressed in the intermediate neural tube and is normally repressed by high levels of Hh signalling. *Dbx1a*, as expected, was ectopically expressed in more ventral positions in embryos in which Hh signalling had been blocked (Fig 8). In the ethanol control embryos *Dbx1a* was noticeably upregulated. However, even though *Dbx1a* was expressed at higher levels in individual cells in ethanol treated embryos, the borders of the *Dbx1a* expression domain were not changed compared to WT domain (Fig 8). Although these data indicate a possible secondary effect of ethanol, which interferes with the level *Dbx1a* expression when applied during early developmental stages, it is evident that Hh signalling is necessary to restrict *Dbx1a* in the intermediate neural tube.

Finally, we looked at the dorsal neural tube marker *Pax3* which we expected to expand ventrally in the absence of Hh signalling. Surprisingly, *Pax3* expression seemed to be unaffected in the absence of Hh signalling (Fig 8). Higher concentrations of cyclopamine (100μM and 200μM) were used to confirm that the effect observed was not due to low dosage of cyclopamine. When 100μM cyclopamine were used zebrafish developed with significant morphological defects and in cases where a properly formed neural tube was obtained *Pax3* expression was maintained. 200μM cyclopamine was very potent for zebrafish which in most cases fail to survive for 24 hours and when they did the development was severely delayed (data not shown).

In addition to cyclopia, all the cyclopamine treated embryos showed U-shaped somites, instead of the normal V-shaped somites seen in WT embryos. This is consistent

with the data of Odenthal et al.. and the requirement for Hh signalling for the induction of muscle pioneer cells and the correct patterning of the paraxial mesoderm(Odenthal et al.., 2000). Cases of other teratogenic effects, including notochord and spinal cord duplications, induced by ethanol exposure have been previously reported (Laale, 1971) but were not observed in our experiments.

To minimize, if possible, the concentration of cyclopamine in subsequent experiments we repeated the analysis using 10μM cyclopamine solution. We found that this concentration produced the same effect as that of 25μM. We concluded that 10μM cyclopamine is sufficient to block Hh signalling (Fig 10 & Fig 11).

Our results provide evidence that blockade of Hh signalling inhibits ventral neural tube patterning in zebrafish in a manner similar to mouse and chick embryos. However, the maintenance of Pax3 expression raises the possibility that some or all dorsal genes in zebrafish may behave in a different manner to Hh signalling than that in the amniotes.

4.2 Temporal requirements for Hh signalling

We next asked what the temporal requirements for Hh signalling are and considered the possibility that different genes might require Hh signalling for different durations. Since hedgehog is thought to act as a morphogen it is possible that Hh signals would have a gradated effect on ventral to dorsal marker expression. In this view, the effect on ventral markers should be more dramatic the earlier in development Hh signalling is blocked. Conversely, the later in development Hh signalling is blocked the less severe the effect would be on the patterning of the neural tube as earlier hedgehog signals would have already induced the expression of some genes. To address this question we blocked Hh signalling at different developmental stages (1 cell stage, 5hpf, 8hpf, 10hpf, 11hpf, 12hpf, 14hpf, 16hpf, 18hpf and 20hpf) using 10μM cyclopamine. Then, by using *in situ*

hybridisation we tested a series of DV markers at 24hpf to assess the temporal requirement of Hh signalling (Table 4 & 5).

We predicted that ventral neural tube markers were expected to be more sensitive to Hh signals at early developmental stages in comparison to intermediate and dorsal markers. To confirm that our observations are due to blockade of Hh signalling and not due to secondary effect of losing expression of the hedgehog genes we also monitored the expression of the zebrafish Hh genes, Shh and Twhh. As development progresses there could be a defined point at which ongoing Hh signalling is no longer required for neural patterning. This would indicate that hedgehog expression is needed for a defined developmental period in order to induce ventral neuronal subtypes. Our predictions on the temporal requirement of shh signalling are summarised in Table 4.

The expression patterns of *Shh* and *Twhh* were not affected by the blockade of Hh signalling at any time point analysed (Fig 22, & Fig 23) indicating that alterations in the expression of genes in the spinal cord were not the consequence of secondary effects of losing expression of hedgehog genes but due to loss of Hh signalling.

Consistent with the complete blockade of Hh signalling described above, the expression of the dorsal neural tube marker *Pax3* was not affected by loss of Hh signalling at any stage during development (Fig 12, 13, 14, and 15). The intermediate marker *Dbx1a* was expanded ventrally when hedgehog expression was disrupted. The expression domain was broader in comparison to that seen in WT zebrafish embryos (Fig 12, 13, 14 and 15). There was a consistent expression of ectopic cells seen in more ventral positions, a result that agreed with our original prediction (Fig 13 & Fig 15).

Blockade of Hh signalling, at any developmental stage between 1 cell stage to 3 somite stage, results in the downregulation, but not complete absence, of *Axial1* expression (Fig 22, 23 and 24). When Hh signalling was blocked at later developmental stages (12hpf)

the expression of *Axial1* was unaffected compared to untreated controls (Fig 25).These data indicate that Axial1 induction and LFP formation requires Hh signalling between 1 cell stage and 6 somite stage (12hpf). After 12hpf Hh signalling is no longer required for the formation of the LFP.

Ventral neural tube markers *Nkx2.2* and *Olig2* failed to be induced when Hh signalling was blocked from early developmental stages. Embryos placed in cyclopamine from 1 cell stage up to 14hpf failed to express both neural tube markers (Fig 17, 18, 19 and 20). However, when embryos were placed in cyclopamine 16hpf *Olig2* expression was detected in the spinal cord in contrast to *Nkx2.2* that was still largely repressed and only a few *Nkx2.2* positive cells at the posterior neural tube were present (Fig 20). When embryos were placed in cyclopamine 18hpf Olig2 expression appeared normal and Nkx2.2 expression was evident in the spinal cord (Fig 21).By 20 hpf both Olig2 and Nkx2.2 expression were indistinguishable from WT embryos (Fig 21). These data indicate that Nkx2.2 and Olig2 are differentially sensitive to the duration of Hh signalling and support the idea that graded Hh signalling is important for in vivo patterning of the ventral neural tube. Finally the ventral neural tube marker, *Nkx6.*1, was downregulated in the absence of Hh signalling when compared to WT expression. However, in contrast to *Nkx2.2* and *Olig2* expression, *Nkx6.1* expression is never lost completely, even when Hh signalling was blocked very early on during development (Fig 16, 17, 18, 19, 20 and 21).

Our results define the temporal requirement of Hh signalling during the development of the zebrafish neural tube. Hedgehog signalling is necessary for the induction of ventral neuronal cell fates and its presence is required for a defined period of time during development for correct neural tube patterning to occur. Table 5 summarises our findings on the temporal requirement of Hh signalling.

4.3 Ventral genes are differentially sensitive to Hh signalling

We next addressed whether different concentrations of cyclopamine differentially affected the expression of ventral neural tube markers. To do this, we placed zebrafish embryos (1 cell stage) in different concentrations of cyclopamine and let them develop for 24 hours. We also repeated the experiment allowing embryos to develop from bud stage (8hpf) in different cyclopamine concentrations and looked at the concentration dependence requirement for Hh signalling in the ventral neural tube.

When zebrafish embryos were placed from 1 cell stage in 3μM and 1μM cyclopamine, partial recovery of the patterning of the neural tube was observed, suggesting that Hh signalling was not completely blocked. *Axial1* was partially recovered at 3μM cyclopamine concentration at the most posterior part of the neural axis (tail tip) (Fig 11), while at 1μM cyclopamine concentration *Axial1* expression was evident in the floorplate along the anterior-posterior axis of the neural tube (Fig 11). At both cyclopamine concentrations (3μM and 1μM) no ectopic Dbx1a positive cells were observed in the ventral neural tube (Fig 10).

At 3μM cyclopamine concentration *Olig2* expression in the head was partially recovered but in the spinal cord Olig2 was absent (Fig 11). At 1μM cyclopamine concentration *Olig2* expression in neural tube was partially recovered with the exception of the most posterior part of the neural tube (Fig 11). In contrast to *Olig2*, *Nkx2.2* expression is not recovered at 3μM cyclopamine (Fig 11) and only some expression in the head is rescued when 1μM cyclopamine concentration was used (Fig 11). These findings suggested the possibility that different genes may require different amounts of Hh signalling to be correctly induced.

We then considered the possibility that the duration of Hh signalling in combination to the strength of the hedgehog signals over a predetermined period of time may be crucial

for ventral neural tube patterning. To test this, zebrafish embryos were allowed to develop from bud stage at a series of cyclopamine concentrations (10μM, 2.5μM, 0.6μM, 0.3μM, 0.2μM and 0.1μM). We considered bud stage to be a critical developmental point because blocking Hh signalling at any time earlier to bud stage showed universal disruption of neuronal markers. Consequently, we focused our attention on analysing the expression patterns of the ventral neural tube markers Nkx6.1, Olig2 and Nkx2.2.

At cyclopamine concentrations 10μM, 2.5μM, 0.6μM and 0.3μM neither Olig2 nor Nkx2.2 were expressed in the spinal cord and expression of Nkx6.1 was disrupted (Fig 26 Fig 27) At a cyclopamine concentration of 0.2μM, Nkx6.1 expression was normal, Olig2 expression was recovered but Nkx2.2 expression was still missing (Fig 27). At a lower cyclopamine concentration of 0.1μM, Nkx6.1 and Olig2 expression was normal and Nkx2.2 expression was also recovered (Fig 27).

These data indicate that ventral genes Nkx6.1, Olig2 and Nkx2.2 are differentially sensitive to Hh signalling. This supports the idea that a combination of the strength and the duration of a signal, Hh signalling, determine the final identity of a progenitor and define the dorsal-ventral organisation of the spinal cord.

TABLE 4

Predictions table indicating the temporal requirement of Shh signalling during zebrafish development.

	1cell stage →					>6somites 12hpf
Pax3	+	+	+	+	+	+
Dbx	-	-	+	+	+	+
Gata2	-	-	-	+	+	+
Nkx6.1	-	-	-	+	+	+
Olig2	-	-	-	-	+	+
Nkx2.2	-	-	-	-	-	+
Axial1	-	+	+	+	+	+
Shh	+	+	+	+	+	+
Twhh	+	+	+	+	+	+

(+) indicates no effect on the neuronal marker and (–) indicates the neuronal marker being disrupted after complete blockage of Hh signalling.

TABLE 5:

Results table showing the temporal requirement of Shh signalling during zebrafish development.

	1cell stage	50%epiboly 5hpf	75%epiboly 8hpf	Bud 10hpf	3somites 11hpf	6somites 12hpf	10somites 14hpf
Pax3	+	+	+	+	+	+	+
Dbx	Ventralisation	Ventralisation	Ventralisation	Ventralisation	Ventralisation	+/-	+
Nkx6.1	+/-	+/-	+/-	+/-	+/-	+/-	+
Olig2	-	-	-	-	-	-	-
Nkx2.2	-	-	-	-	-	-	-
Axial1	+/-	+/-	+/-	+/-	+/-	+	+
Shh	+	+	+	+	+	+	+
Twhh	+	+	+	+	+	+	+

Hh signalling was blocked by the use of cyclopamine at different developmental stages. (+) indicates no effect on the neuronal marker, (–) indicates total loss of the neuronal marker and (+/-) indicates that the marker appeared downregulated.

Note

Experiments were repeated 3 times (minimum).

Number of embryos per experiment: 10

100% of embryos showed the phenotype described

Figure 8: 25μM cyclopamine, previously shown to be sufficient to block Hh signalling, has no effect on the dorsal neural tube marker *Pax3* (C) but it interferes with patterning of the intermediate neural tube as shown by the expression pattern of *Dbx* (F), which expands ventrally. Zebrafish embryos were placed in cyclopamine solution at 1 cell stage and allowed to develop until 24hpf before fixation and *in situ* hybridization assay for the indicated genes.

Figure 9: 25μM cyclopamine has a severe effect on ventral neural tube patterning as shown by the loss of the ventral neural tube markers *Olig2* (C) and *Nkx2.2* (F) and the downregulation of the floor plate marker *Axial1* (I). Zebrafish embryos were placed in cyclopamine solution at 1 cell stage and allowed to develop until 24hpf before fixation and *in situ* hybridization assay for the indicated genes.

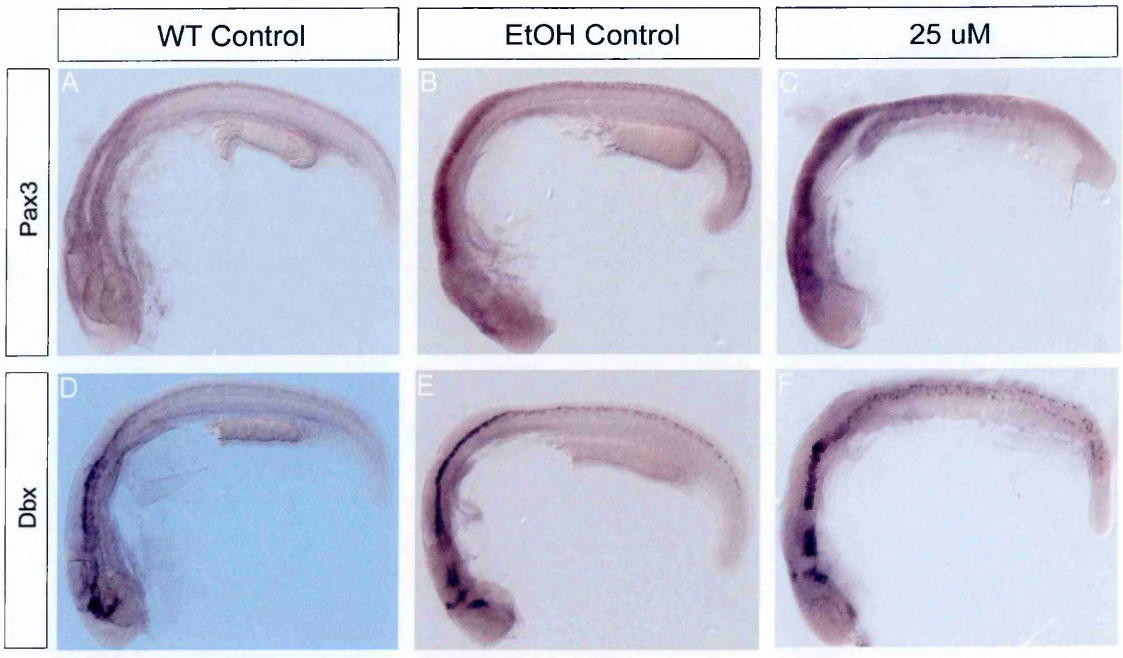

Figure 8: Blockade of Hh signalling using cyclopamine does not affect patterning of the dorsal neural tube but it interferes with intermediate neural tube patterning. [Dorsal (top) – Ventral (bottom)]

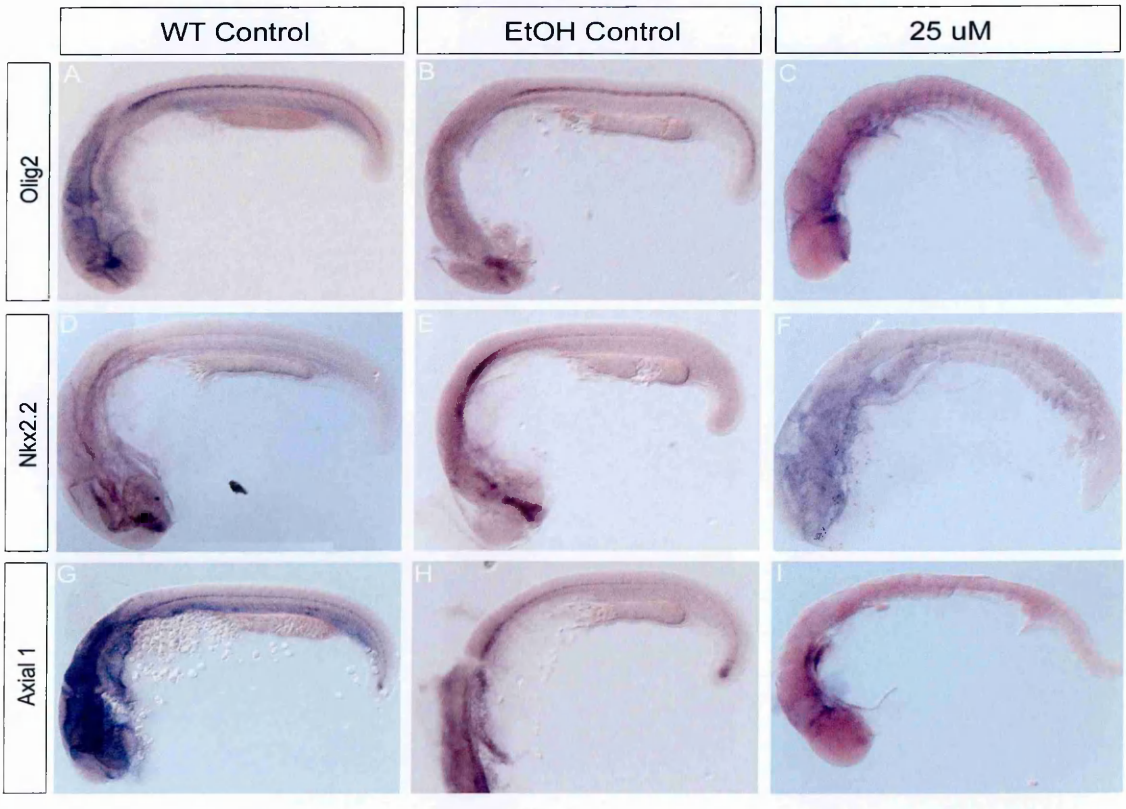

Figure 9: Blockade of Hh signalling using cyclopamine has a severe effect on ventral neural tube patterning [Dorsal (top) – Ventral (bottom)]

Figure 10: 10μM cyclopamine is sufficient to block Hh signalling. Similar to 25μM cyclopamine, 10μM cyclopamine does not affect the dorsal neural tube marker *Pax3* (A) but the patterning of the intermediate neural tube marker *Dbx* (D) is affected. Both markers were unaffected when 3μM (B & E) and 1μM (C & F) cyclopamine was used. Zebrafish embryos were placed in cyclopamine solution at 1 cell stage and allowed to develop until 24hpf before fixation and *in situ* hybridization assay for the indicated genes. (*In situ* hybridisation)

Figure 11: 10μM cyclopamine, similar to 25μM cyclopamine, has a severe effect on ventral neural tube patterning as shown by the loss of the ventral neural tube markers *Olig2* (A) and *Nkx2.2* (D) and the downregulation of the floor plate marker *Axial1* (G). 3μM cyclopamine also resulted in the loss of *Olig2* (B) and *Nkx2.2* (E) while *Axial1* is now present at the tail tip (arrow) (H). 1μM cyclopamine allowed recovery of *Olig2* (C) and *Axial1* (I) expression but *Nkx2.2* (F) was still absent. Zebrafish embryos were placed in cyclopamine solution at 1 cell stage and allowed to develop until 24hpf before fixation and *in situ* hybridization assay for the indicated genes. . (*In situ* hybridisation)

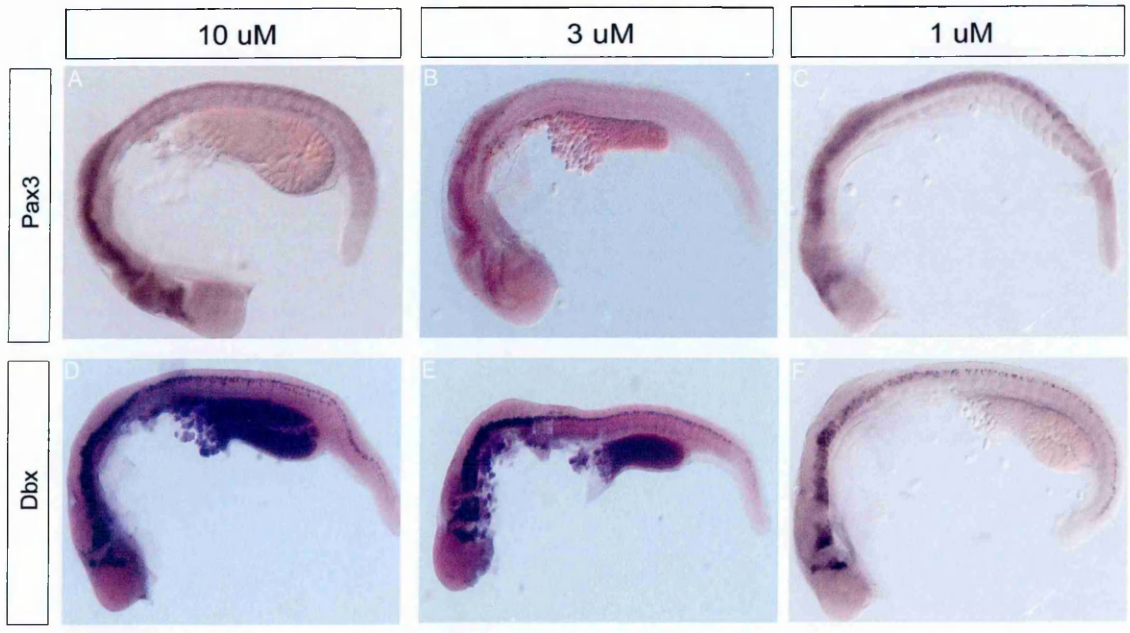

Figure 10: 10μM of cyclopamine is sufficient to block Hh signalling (A) [Dorsal (top) – Ventral (bottom)]

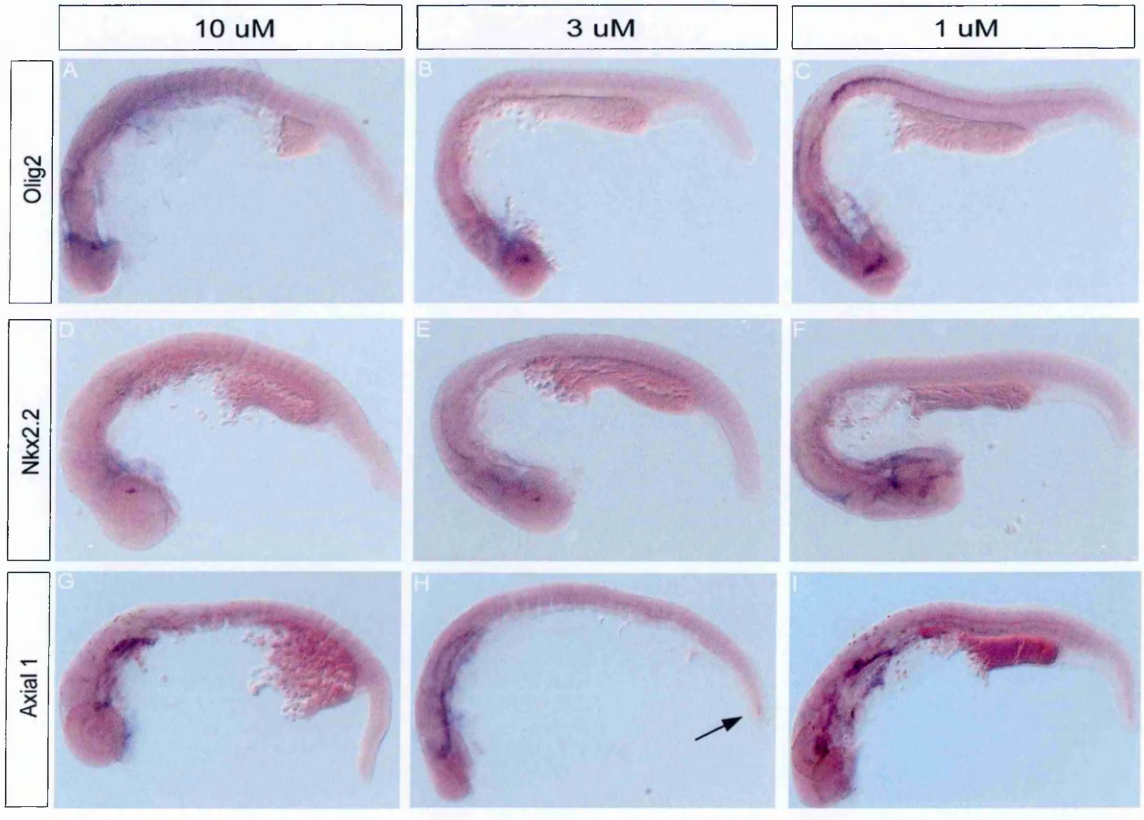

Figure 11: 10μM of cyclopamine is sufficient to block Hh signalling (B) [Dorsal (top) – Ventral (bottom)]

Figure 12: Expression pattern of the dorsal neural tube marker *Pax3* and the intermediate marker *Dbx* in wild type [A (a) and C (c)] and 0.1% ethanol treated embryos [B (b) and D (d)]. Ethanol treatment had no effect on the expression of these spinal cord genes. Zebrafish embryos were placed in ethanol solution at 1 cell stage and allowed to develop until 24hpf before fixation and *in situ* hybridization assay for the indicated genes. (*In situ* hybridisation)

Figure 13: Zebrafish placed in 10μM cyclopamine at 1 cell stage or 50% epiboly. There was no effect on the expression of the dorsal neural tube marker *Pax3* [A (a) & B (b)]. The intermediate neural tube marker *Dbx* [C (c) & D (d, arrow)] was affected by the loss of Hh signalling at both time points as seen by the ectopic expression of Dbx in more ventral positions. Zebrafish embryos were placed in cyclopamine solution at 1 cell stage or 50% epiboly and allowed to develop until 24hpf before fixation and *in situ* hybridization assay for the indicated genes. (*In situ* hybridisation)

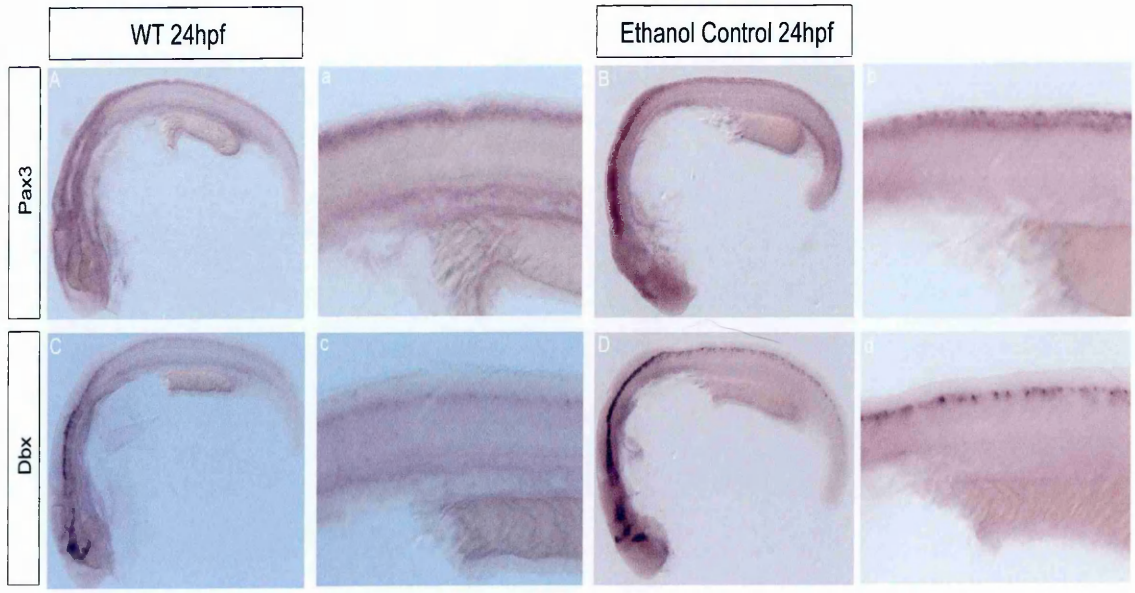

Figure 12: 0.1% Ethanol does not affect D-V patterning of the neural tube (A) [Dorsal (top) – Ventral (bottom)]

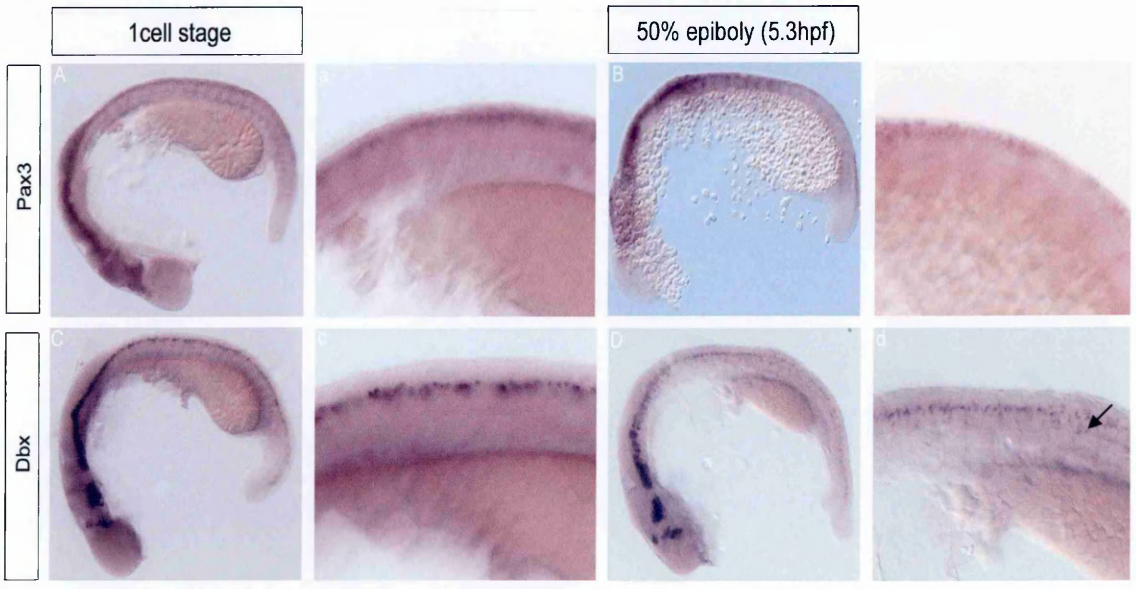

Figure 13: Zebrafish placed in 10μM cyclopamine at 1 cell stage and 50% epiboly (A) [Dorsal (top) – Ventral (bottom)]

Figure 14: Zebrafish placed in 10μM cyclopamine at 75% epiboly and at bud stage. There was consistently no effect on the dorsal neural tube marker *Pax3* [A (a) & B (b)]. The intermediate neural tube marker *Dbx* [C (c) & D (d)] was affected by the loss of Hh signalling as seen by the broader expression domain of Dbx compared to WT embryos. Zebrafish embryos were placed in cyclopamine solution at 75% epiboly or at bud stage and allowed to develop until 24hpf before fixation and *in situ* hybridization assay for the indicated genes. (*In situ* hybridisation)

Figure 15: Zebrafish placed in 10μM cyclopamine at 3 and 6 somite stage. No effect was observed on the dorsal neural tube marker *Pax3* [A (a) & B (b)]. The intermediate neural tube marker *Dbx* [C (c) & D (d)] was affected by the loss of Hh signalling as seen by the ectopic expression of Dbx positive cells in more ventral position. Zebrafish embryos were placed in cyclopamine solution at 3 or 6 somite stage and allowed to develop until 24hpf before fixation and *in situ* hybridization assay for the indicated genes. (*In situ* hybridisation)

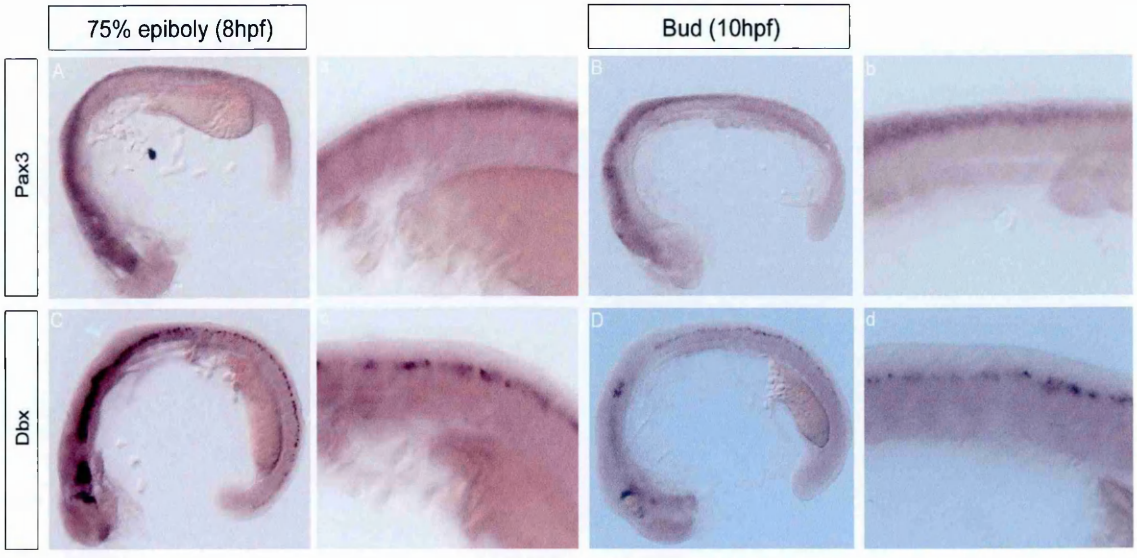

Figure 14: Zebrafish placed in 10μM cyclopamine at 75% epiboly and at bud stage (A) [Dorsal (top) – Ventral (bottom)]

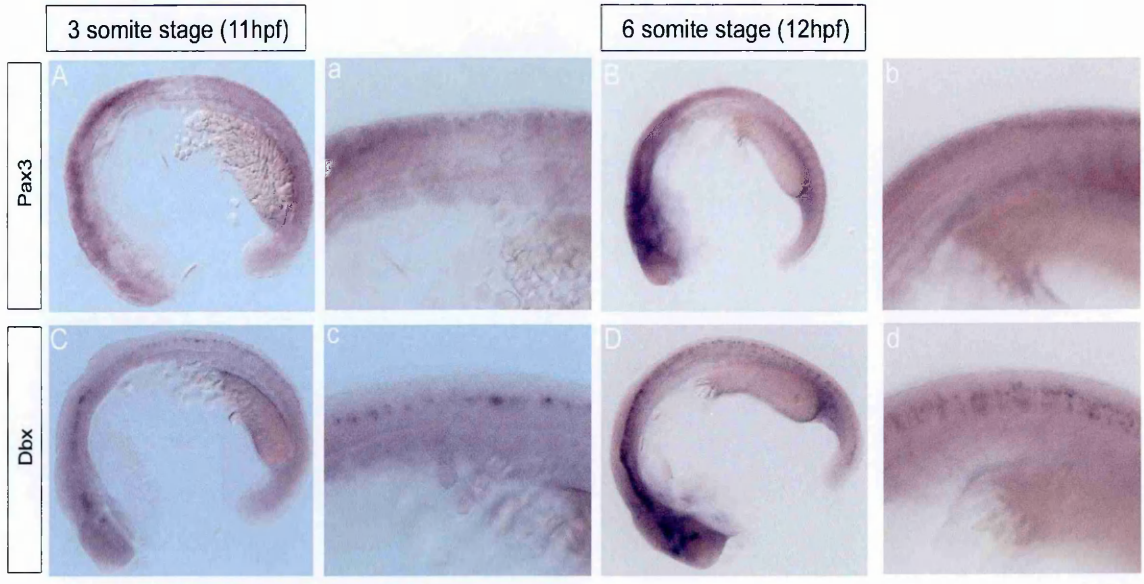

Figure 15: Zebrafish placed in 10μM cyclopamine at 3 and 6 somite stage (A) [Dorsal (top) – Ventral (bottom)]

Figure 16: The ventral neural tube markers *Nkx6.1*, *Olig2* and *Nkx2.2* [A (a), C (c) & E (e)] were similarly expressed in untreated and ethanol treated embryos [B (b), D (d) & F (f)] indicating that that ethanol treatment had no effect on spinal cord patterning. Zebrafish embryos were placed in cyclopamine solution at 1 cell stage and allowed to develop until 24hpf before fixation and *in situ* hybridization assay for the indicated genes. (*In situ* hybridisation)

Figure 17: When zebrafish were placed in 10μM cyclopamine at 1 cell stage and 50% epiboly, the expression of the ventral neural tube markers *Olig2* [C (c) & D (d)] and *Nkx2.2* [E (e) & F (f)] was lost. *Nkx6.1* [A (a) & B (b)] was also affected as seen by its downregulation in treated embryos. Zebrafish embryos were placed in cyclopamine solution at 1 cell stage or 50% epiboly and allowed to develop until 24hpf before fixation and *in situ* hybridization assay for the indicated genes. (*In situ* hybridisation)

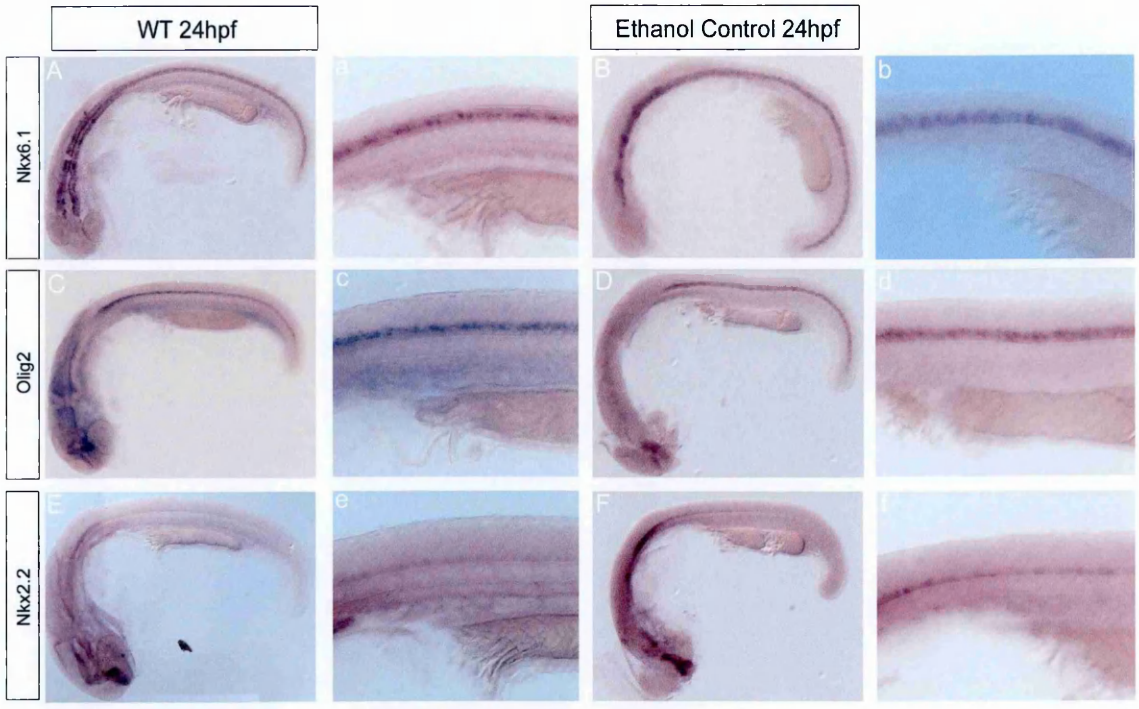

Figure 16: 0.1% Ethanol does not affect D-V patterning of the neural tube (B) [Dorsal (top) – Ventral (bottom)]

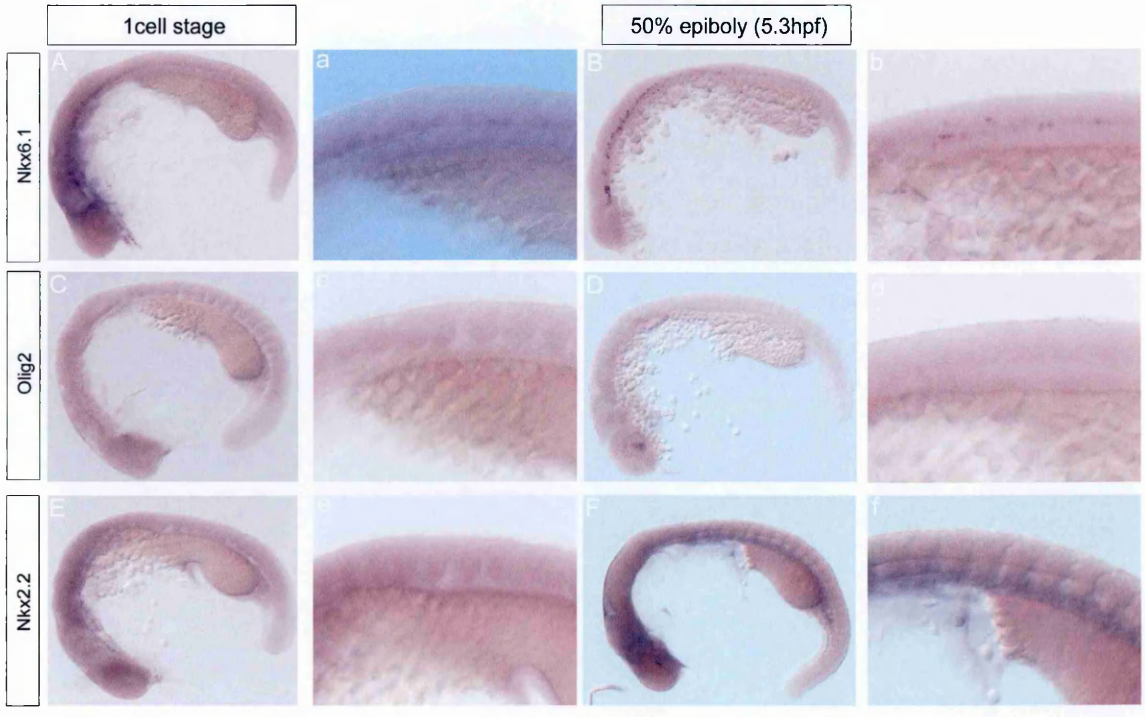

Figure 17: Zebrafish placed in 10μM cyclopamine at 1 cell stage and 50% epiboly (B) [Dorsal (top) – Ventral (bottom)]

Figure 18: When zebrafish were placed in 10μM cyclopamine at 75% epiboly and at bud stage, the expression of the ventral neural tube markers *Olig2* [C (c) & D (d)] and *Nkx2.2* [E (e) & F (f)] was lost. *Nkx6.1* [A (a) & B (b)] was also affected as seen by its downregulation in treated embryos. Zebrafish embryos were placed in cyclopamine solution at 75% epiboly or at bud stage and allowed to develop until 24hpf before fixation and *in situ* hybridization assay for the indicated genes. (*In situ* hybridisation)

Figure 19: When zebrafish were placed in 10μM cyclopamine at 3 and 6 somite stage, the expression of the ventral neural tube markers *Olig2* [C (c) & D (d)] and *Nkx2.2* [E (e) & F (f)] was lost. *Nkx6.1* [A (a) & B (b)] was also affected as seen by its downregulation in treated embryos. Zebrafish embryos were placed in cyclopamine solution at 3 or 6 somite stage and allowed to develop until 24hpf before fixation and *in situ* hybridization assay for the indicated genes. (*In situ* hybridisation)

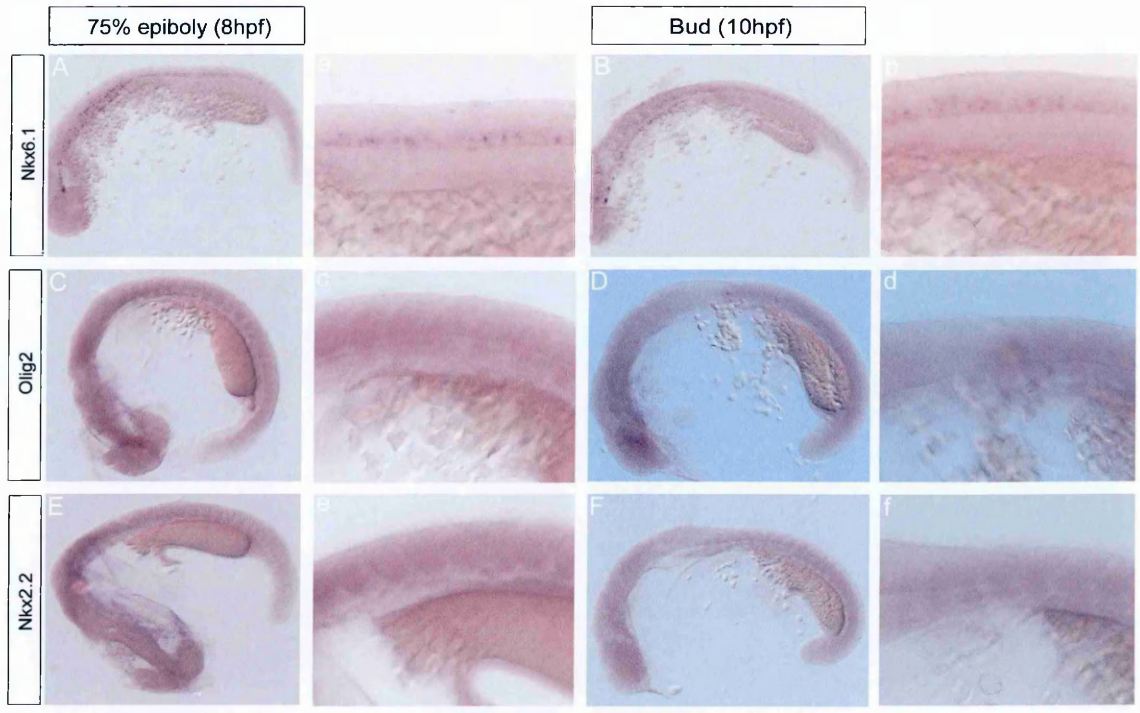

Figure 18: Zebrafish placed in 10μM cyclopamine at 75% epiboly and at bud stage (B) [Dorsal (top) – Ventral (bottom)]

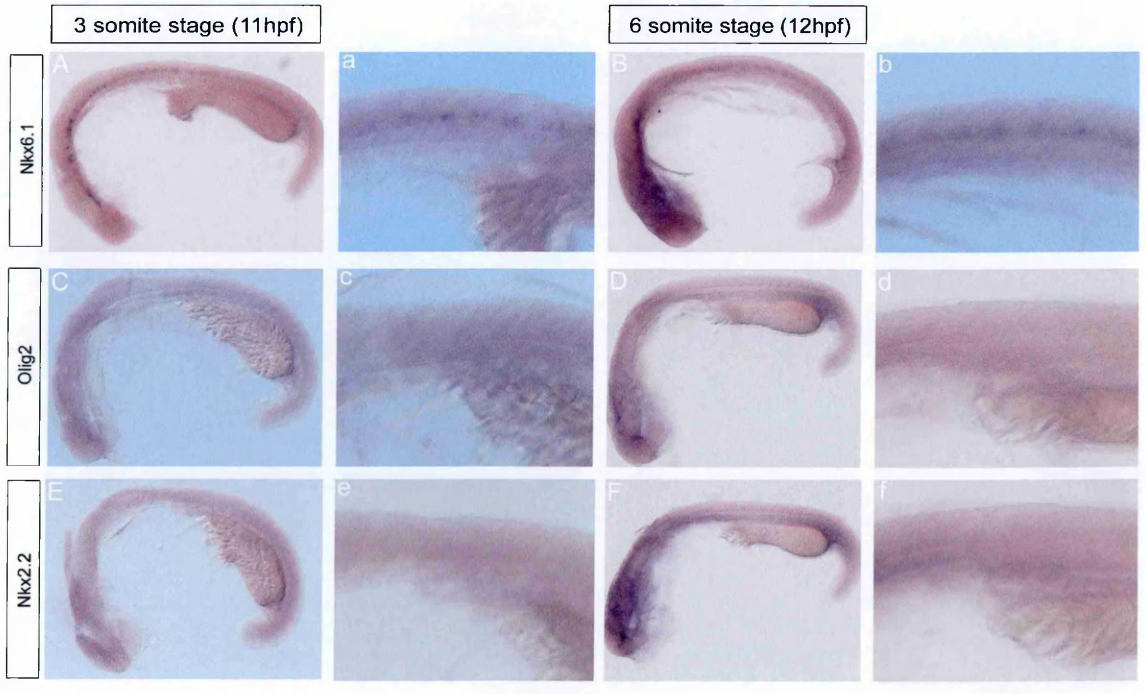

Figure 19: Zebrafish placed in 10μM cyclopamine at 3 and 6 somite stage (B) [Dorsal (top) – Ventral (bottom)]

Figure 20: When zebrafish were placed in 10μM cyclopamine at 10 somite stage, the expression of the ventral neural tube markers *Olig2* [C (c)] and *Nkx2.2* [E (e)] was lost. *Nkx6.1* [A (a)] was also affected as seen by its downregulation in treated embryos. However, when zebrafish were placed in 10μM cyclopamine at 14 somite stage, expression of the ventral neural tube marker *Olig2* [D (d)] was detectable in contrast to the *Nkx2.2* [F (f)] that was still largely repressed, while *Nkx6.1* [B (b)] appeared normal. Zebrafish embryos were placed in cyclopamine solution at 10 or 14 somite stage and allowed to develop until 24hpf before fixation and *in situ* hybridization assay for the indicated genes. (*In situ* hybridisation)

Figure 21: When zebrafish were placed in 10μM cyclopamine at 18 somite stage, the ventral neural tube marker *Olig2* [C (c)] appeared normal, *Nkx2.2* expression [E (e)] was now evident in the ventral neural tube and *Nkx6.1* [A (a)] was unaffected. When zebrafish were placed in 10μM cyclopamine at 21 somite stage, all three markers appeared normal [B (b), D (d) & F (f)] Zebrafish embryos were placed in cyclopamine solution at 18 or 21 somite stage and allowed to develop until 24hpf before fixation and *in situ* hybridization assay for the indicated genes. (*In situ* hybridisation)

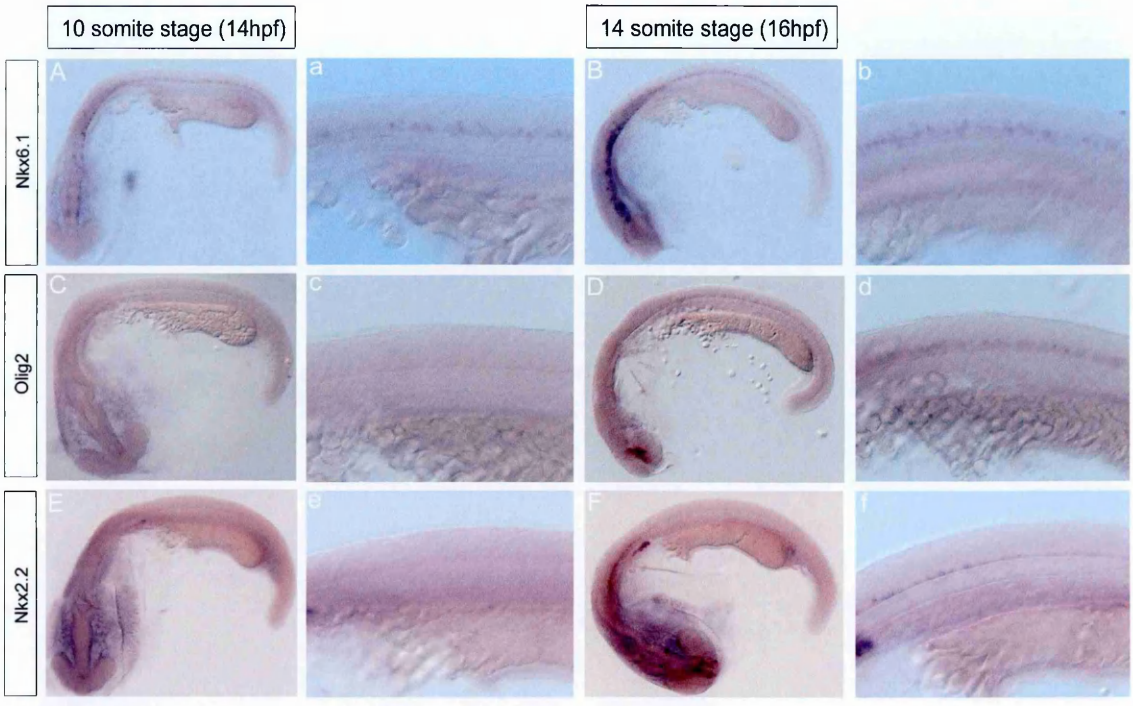

Figure 20: Zebrafish placed in 10μM cyclopamine at 10 and 14 somite stages [Dorsal (top) – Ventral (bottom)]

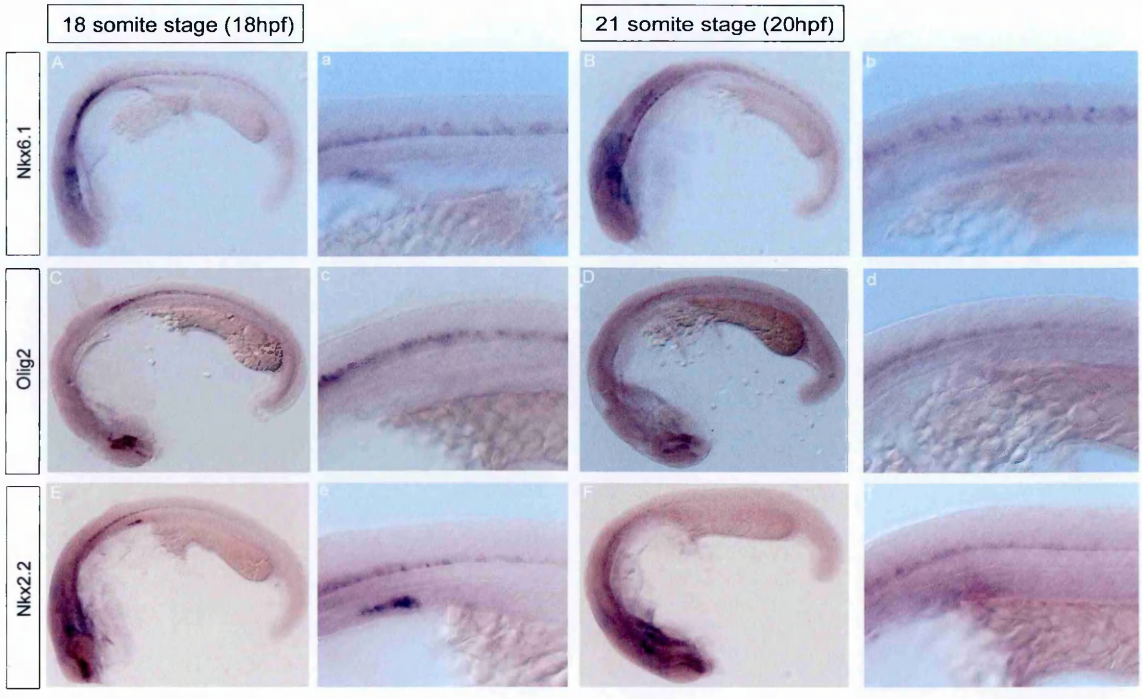

Figure 21: Zebrafish placed in 10μM cyclopamine at 18 and 21 somite stages [Dorsal (top) – Ventral (bottom)]

Figure 22: The expression of the Hh genes Shh & Twhh as well as the floor plate marker *Axial1* in wild type [C (c), E (e) & A (a)] and 0.1% ethanol treated embryos [D (d), F (f) & B (b)] showed that ethanol treatment has no effect on floor plate markers. Zebrafish embryos were placed in cyclopamine solution at 1 cell stage and allowed to develop until 24hpf before fixation and *in situ* hybridization assay for the indicated genes. (*In situ* hybridisation)

Figure 23: Zebrafish placed in 10μM cyclopamine at 1 cell stage and 50% epiboly. Expression of the Hh genes Shh [C (c) & D (d)] and Twhh [E (e) & F (f)] were unaffected by the loss of Hh signalling. *Axial1* [A (a) & B (b)] was downregulated but not absent. Zebrafish embryos were placed in cyclopamine solution at 1 cell stage or 50% epiboly and allowed to develop until 24hpf before fixation and *in situ* hybridization assay for the indicated genes. (*In situ* hybridisation)

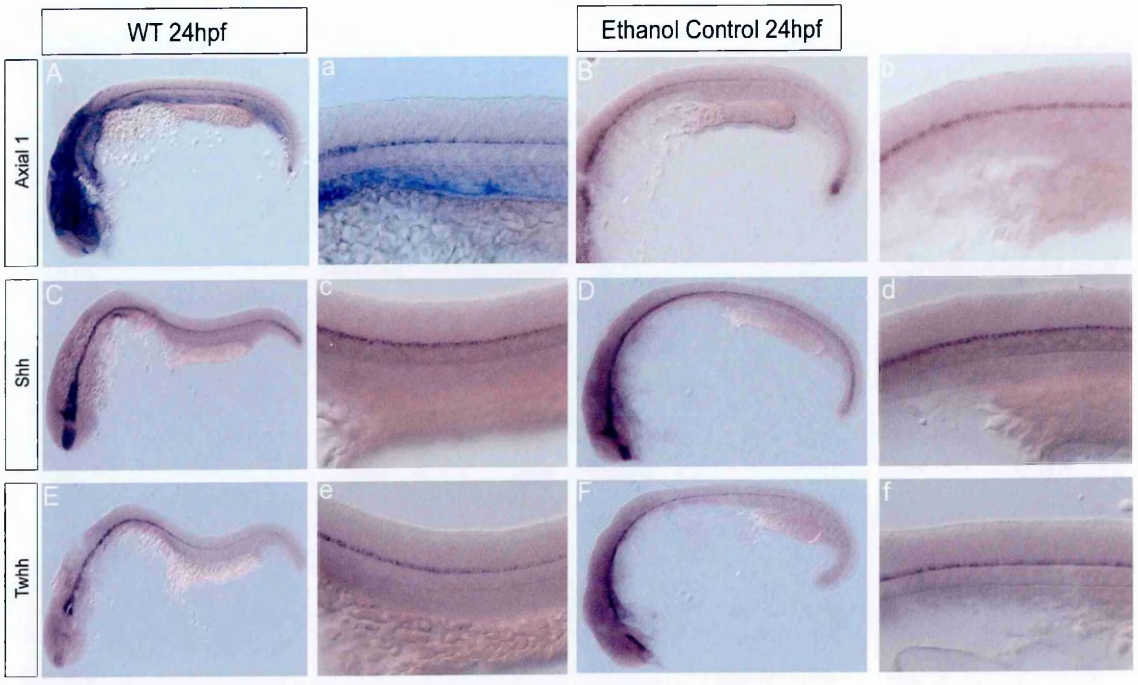

Figure 22: 0.1% Ethanol does not affect D-V patterning of the neural tube (C) [Dorsal (top) – Ventral (bottom)]

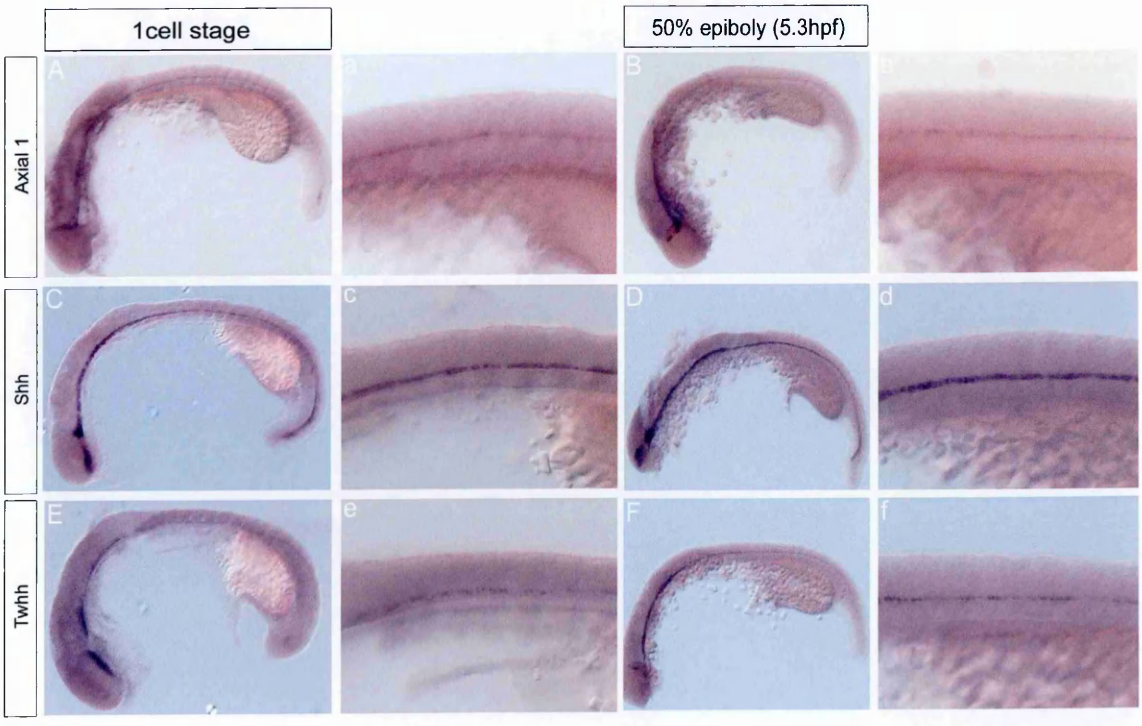

Figure 23: Zebrafish placed in 10μM cyclopamine at 1 cell stage and 50% epiboly (C) [Dorsal (top) – Ventral (bottom)]

Figure 24: Zebrafish placed in 10μM cyclopamine at 75% epiboly and at bud stage. Expression of the Hh genes Shh [C (c) & D (d)] and Twhh [E (e) & F (f)] were unaffected by the loss of Hh signalling. *Axial1* [A (a) & B (b)] was downregulated but not absent. Zebrafish embryos were placed in cyclopamine solution at 75% epiboly or at bud stage and allowed to develop until 24hpf before fixation and *in situ* hybridization assay for the indicated genes. (*In situ* hybridisation)

Figure 25: When zebrafish were placed in 10μM cyclopamine at 3 somite stage, the Hh markers *Shh* [C (c)] and *Twhh* [E (e)] were unaffected by the loss of Hh signalling. *Axial1* [A (a)] was downregulated but not absent. However, when zebrafish were placed in cyclopamine at 6 somite stage all three markers appeared normal [B (b), D (d) & F (f)]. Zebrafish embryos were placed in cyclopamine solution at 3 or 6 somite stage and allowed to develop until 24hpf before fixation and *in situ* hybridization assay for the indicated genes. (*In situ* hybridisation)

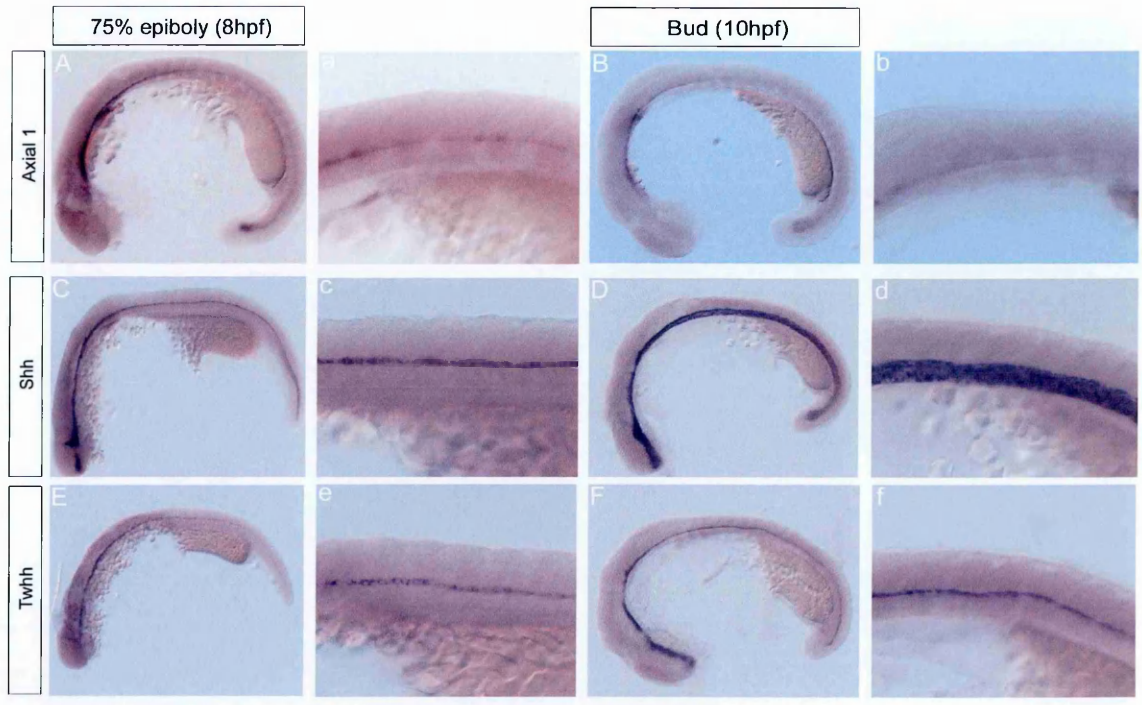

Figure 24: Zebrafish placed in 10μM cyclopamine at 75% epiboly and at bud stage (C) [Dorsal (top) – Ventral (bottom)]

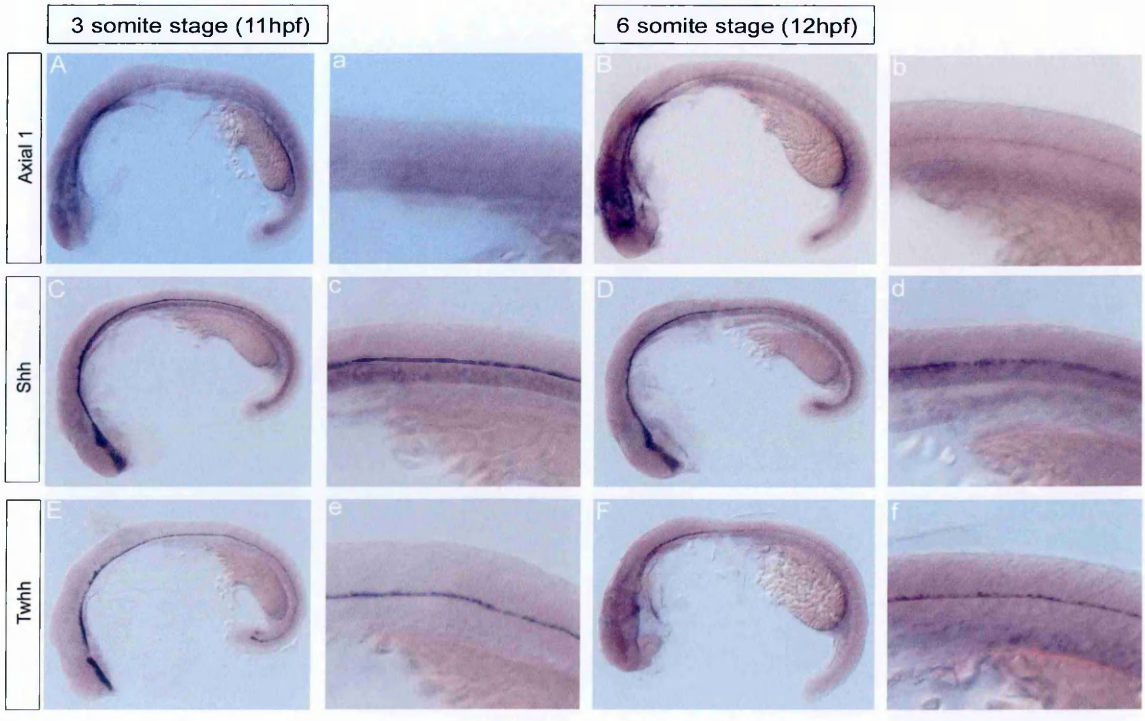

Figure 25: Zebrafish placed in 10μM cyclopamine at 3 and 6 somite stage (C) [Dorsal (top) – Ventral (bottom)]

Figure 26: Different cyclopamine concentrations (10μM, 2.5μM, 0.6μM) were used to assess whether ventral genes were differentially sensitive to Hh signalling. Consistent with our previous results (Fig 17) *Olig2* (D, E & F) and *Nkx2.2* (G, H & I) were lost from the ventral region of the spinal cord while *Nkx6.1* (A, B & C) expression was disrupted when 10μM, 2.5μM and 0.6μM cyclopamine was used. Zebrafish embryos were placed in cyclopamine solutions at bud stage and allowed to develop until 24hpf before fixation and *in situ* hybridization assay for the indicated genes. (*In situ* hybridisation)

Figure 27: Different cyclopamine concentrations (0.3μM, 0.2μM, 0.1μM) were used to assess whether ventral genes were differentially sensitive to Hh signalling When 0.3μM of cyclopamine was used *Olig2* (D, E & F) and *Nkx2.2* (G, H & I) were lost from the ventral region of the spinal cord while *Nkx6.1* (A, B & C) expression was disrupted. At a cyclopamine concentration of 0.2μM, *Nkx6.1* (H) expression was normal, *Olig2* (E) expression was recovered but *Nkx2.2* (H) expression was still missing. At a lower cyclopamine concentration of 0.1μM, *Nkx6.1* (C) and *Olig2* (F) expression was normal and *Nkx2.2* (I) expression was also recovered. Zebrafish embryos were placed in cyclopamine solutions at bud stage and allowed to develop until 24hpf before fixation and *in situ* hybridization assay for the indicated genes. (*In situ* hybridisation)

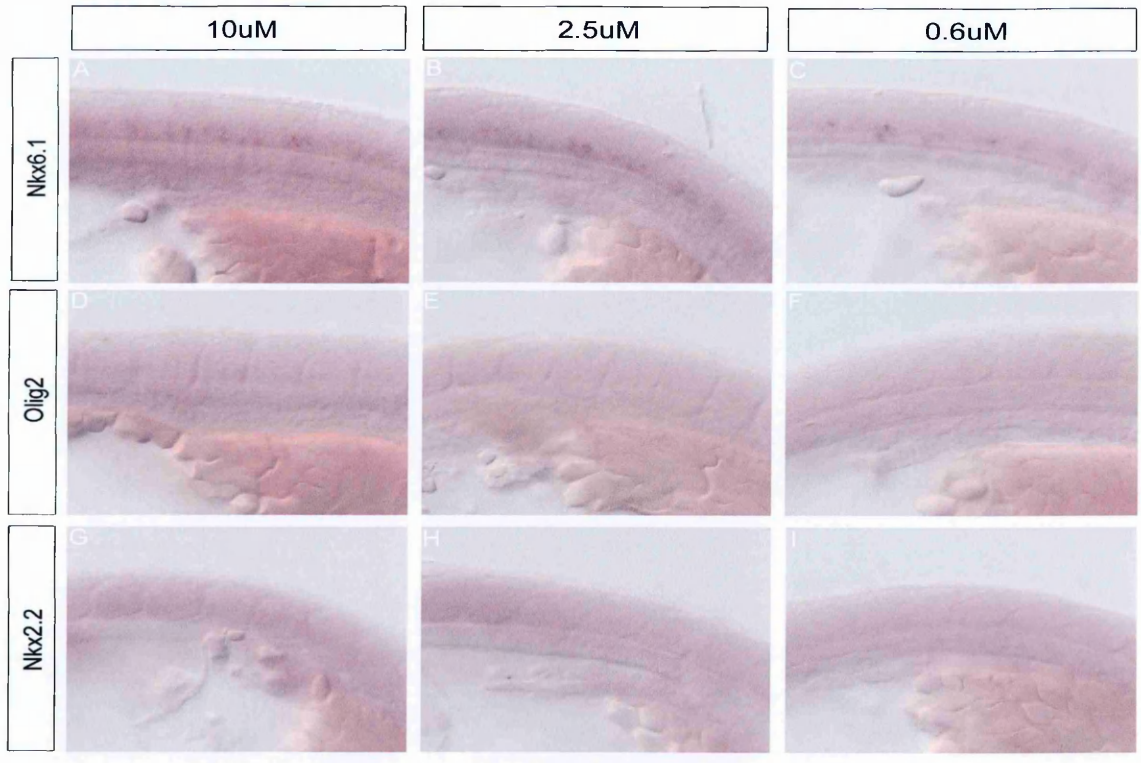

Figure 26: Ventral genes are differentially sensitive to Hh signalling (A) [Dorsal (top) – Ventral (bottom)]

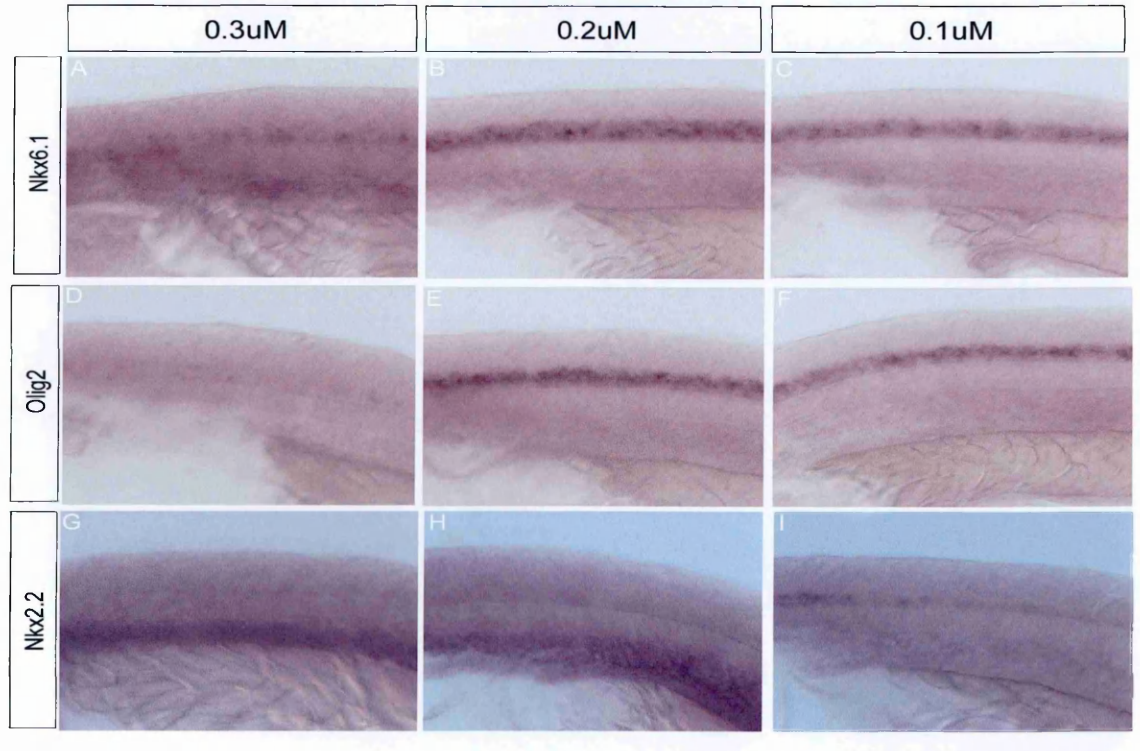

Figure 27: Ventral genes are differentially sensitive to Hh signalling (B) [Dorsal (top) – Ventral (bottom)]

5. Results

5.1 Generation of Nkx2.2 and Pax6 mouse double mutant (Nkx2.2/Pax6 $^{-/-}$) and analysis of the neural patterning in the spinal cord and hindbrain.

The homeodomain proteins Nkx2.2 and Pax6 are expressed in progenitor cell populations of the ventral neural tube and respond to graded Shh signalling (Briscoe et al.., 1999; Ericson et al.., 1997b). Nkx2.2 (class II protein) is dependent on Shh signalling while Pax6, (class I protein) is repressed by Shh signalling (Briscoe et al.., 2000). The cross-repressive interactions between these two proteins have been proposed to establish sharp boundaries needed for correct gene expression and consequent neuronal patterning (Briscoe et al.., 2000). Nkx2.2$^{-/-}$ (Sussel et al.., 1998) and Pax6$^{-/-}$ (Hill et al.., 1991) mice have been previously reported and the role of both genes during development has been extensively studied (Briscoe and Ericson, 2001; Briscoe et al.., 2000; Briscoe et al.., 1999; Ericson et al.., 1997b). However, Nkx2.2/Pax6 $^{-/-}$ mice have not been reported to date.

Originally, we crossed Nkx2.2$^{+/-}$ with Pax6$^{+/-}$ mice and obtained the mutant strain Nkx2.2/Pax6$^{+/-}$ (Fig 28). However when mice of this strain were mated together, no Nkx2.2/Pax6$^{-/-}$ embryos were obtained (>120 embryos collected and genotyped). This could either be because (a) double mutant homozygous embryos are embryonic lethal or (b) that the two genes are located close together on the same chromosome. Mouse genome information was limited at the beginning of the project but as the genome became available it became apparent that Nkx2.2 and Pax6 were located on the same chromosome. Both

Nkx2.2 and Pax6 are located on Chromosome 2 in the mouse genome and are ~41 Mb apart. To calculate the recombination frequency needed to obtain the desired offspring we considered the theoretical genetic distance between these two genes. The genetic distance is measured in centimorgans (cM) with one centimorgan defined as the distance between two loci that recombine with a frequency of 1% (http://www.informatics.jax.org). In the mouse on average 1 megabase (1Mb) is equivalent to 0.5cM (http://www.informatics.jax.org). Thus, the crossover frequency of acquiring the desired offspring, Nkx2.2/Pax6$^{-/-}$, when mating two Nkx2.2/Pax6$^{+/-}$ mice was only 1% (Fig 28, Cross B).

To increase the probability of generating the desired double mutant we adopted an alternative strategy. We crossed WT mice with double heterozygote mutants and identified pups containing both mutant alleles (Fig 28, Cross C). Animals from these litters containing both mutant alleles must have undergone a crossover event between Nkx2.2 and Pax6. Genotyping of pups from these matings indicated that the crossover frequency was ~5%. Animals with both mutant alleles on the same chromosome were then used to develop an Nkx2.2/Pax6$^{+/-}$ colony (mutant alleles of both genes on the same chromosome) and these produced double heterozygote animals at a higher frequency of ~25% (Fig 28, Cross D). These mice were then crossed together to produce the desired double mutant Nkx2.2/Pax6$^{-/-}$ (Fig 28, Cross E).

We then analysed neural patterning of the double mutant in the spinal cord and hindbrain. Based on the analysis and interpretation of the single mutants we formulated a model of neural patterning that predicted the changes expected in the absence of both Nkx2.2 and Pax6 (Fig 29). In the spinal cord, the absence of Pax6 results in the dorsal expansion of Nkx2.2 (Ericson et al.., 1997b). In Pax6$^{-/-}$ embryos, this dorsal expansion of Nkx2.2 correlates with the dorsal expansion in V3 neurons and the loss of somatic motor neurons. (Briscoe et al.., 1999; Ericson et al.., 1997b). In Pax6 mutants there is also

reduction in V2 neurons and loss of V1 neurons, both of which derive from Pax6 progenitor cells (Ericson et al.., 1997b). In contrast, loss of Nkx2.2 does not affect Pax6 expression (Briscoe et al.., 1999). However, in Nkx2.2-/- embryos there is a ventral expansion of somatic motor neuron (sMN) generation and a subsequent loss of V3 neurons derived from Nkx2.2 progenitor cells (Briscoe et al.., 1999). These observations led us to predict that in the spinal cord in the absence of both Nkx2.2 and Pax6 (Nkx2.2/Pax6$^{-/-}$) we would expect loss of V3 neurons and a ventral expansion of somatic MNs as a consequence of the absence of Nkx2.2. Loss of V1 neurons due to absence of Pax6 expression is also expected. This phenotype resembles most closely the Nkx2.2$^{-/-}$ phenotype (Fig 29).

In the hindbrain, the ventral-most progenitor cells give rise to visceral motor neurons (vMN) instead of V3 neurons (Ericson et al.., 1997b). The generation of vMN in Nkx2.2$^{-/-}$ embryos is similar to WT mice, indicating that Nkx2.2 is not required for visceral MN generation (Briscoe et al.., 1999). It was suggested that Nkx2.9 expression could account for these observations (Briscoe et al.., 1999). Nkx2.9 is closely related to Nkx2.2 and expressed in the same domain as Nkx2.2 moreover its expression persists in the hindbrain in contrast to its downregulation in the spinal cord (Briscoe et al.., 1999). Thus, Nkx2.9 activity may compensate for the loss of Nkx2.2 (Briscoe et al.., 1999). In Pax6$^{-/-}$ embryos, visceral MNs expand dorsally, correlating with the dorsal expansion of Nkx2.2 and consequently the generation of somatic MNs is inhibited. Similar to the spinal cord, there is also repression of V2 neurons and loss of V1 neurons (Ericson et al.., 1997b). The presence of Nkx2.9 in the hindbrain led us to predict that the hindbrain phenotype of the Nkx2.2/Pax6$^{-/-}$ mice would resemble that of Pax6$^{-/-}$ mutant. The loss of Pax6 would result in a dorsal expansion of visceral MNs and repression of somatic MNs. We would also observe reduction of V2 interneurons and loss of V1 neurons (Fig 30).

To test our hypothesis, we used immunohistochemistry and *in situ* hybridisation to assay a series of progenitor and neuronal markers in the Nkx2.2/Pax6$^{-/-}$ mutants. Nkx2.2$^{-/-}$, Pax6$^{-/-}$ and WT mice were used as controls. To confirm that we had successfully generated the desired double mutant mice we firstly looked at the spinal cord and the hindbrain expression of Nkx2.2 and Pax6 in E10.5 and E11.5 WT, Nkx2.2$^{-/-}$, Pax6$^{-/-}$ and Nkx2.2/Pax6$^{-/-}$ mice. As expected, in WT embryos Nkx2.2 is expressed in the ventral-most region of the neural tube while Pax6 is expressed dorsal to Nkx2.2 (Fig 31). In Nkx2.2$^{-/-}$ the Pax6 expression remains unchanged while in Pax6$^{-/-}$ there is a dorsal expansion of Nkx2.2 (Fig 31). Finally, in the Nkx2.2/Pax6$^{-/-}$ we observed loss of both progenitor markers (Fig 31). This confirms that we had successfully generated the double mutant line.

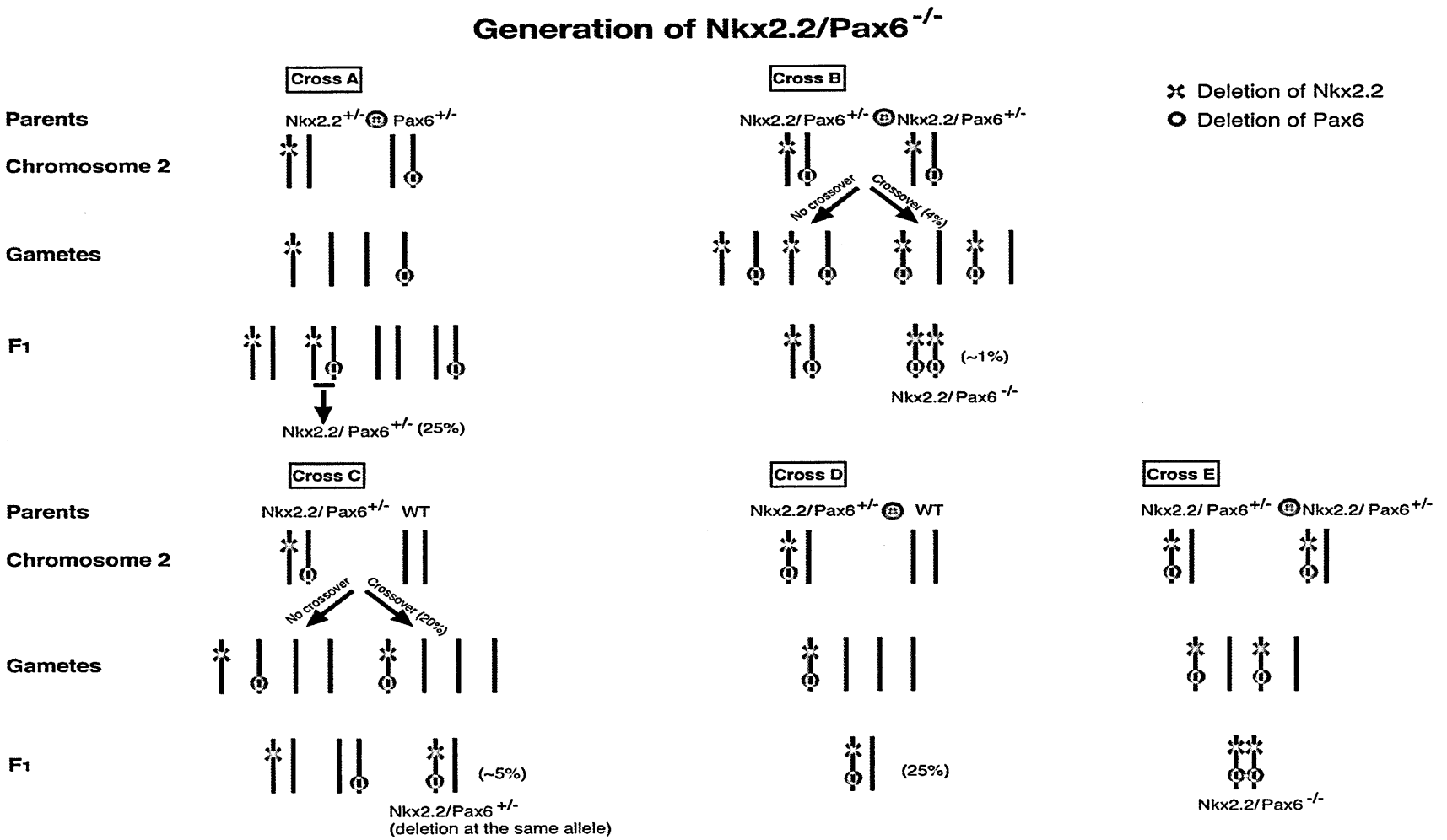

Figure 28 Diagram showing the mouse matings resulted in the generation of the double mutant mouse (Nkx2.2/Pax6-/-).

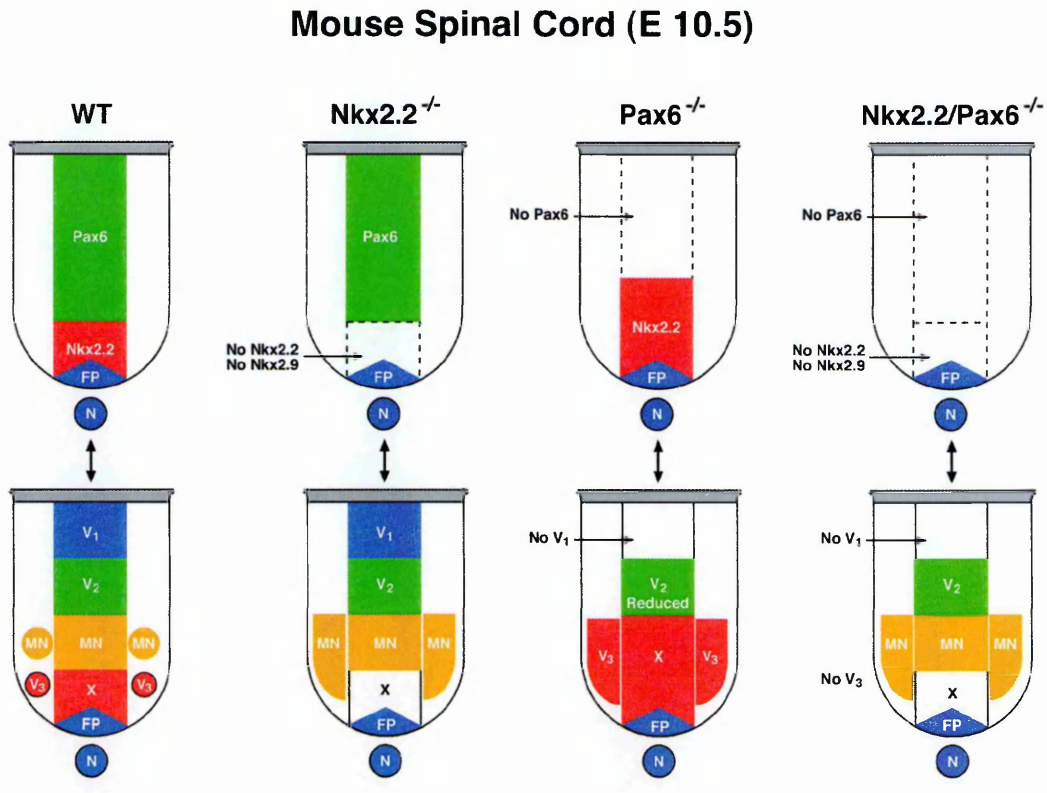

Figure 29 Schematic showing the patterning of the ventral neural tube of the single and double mutants (spinal cord level). [Dorsal (top) – Ventral (bottom)]

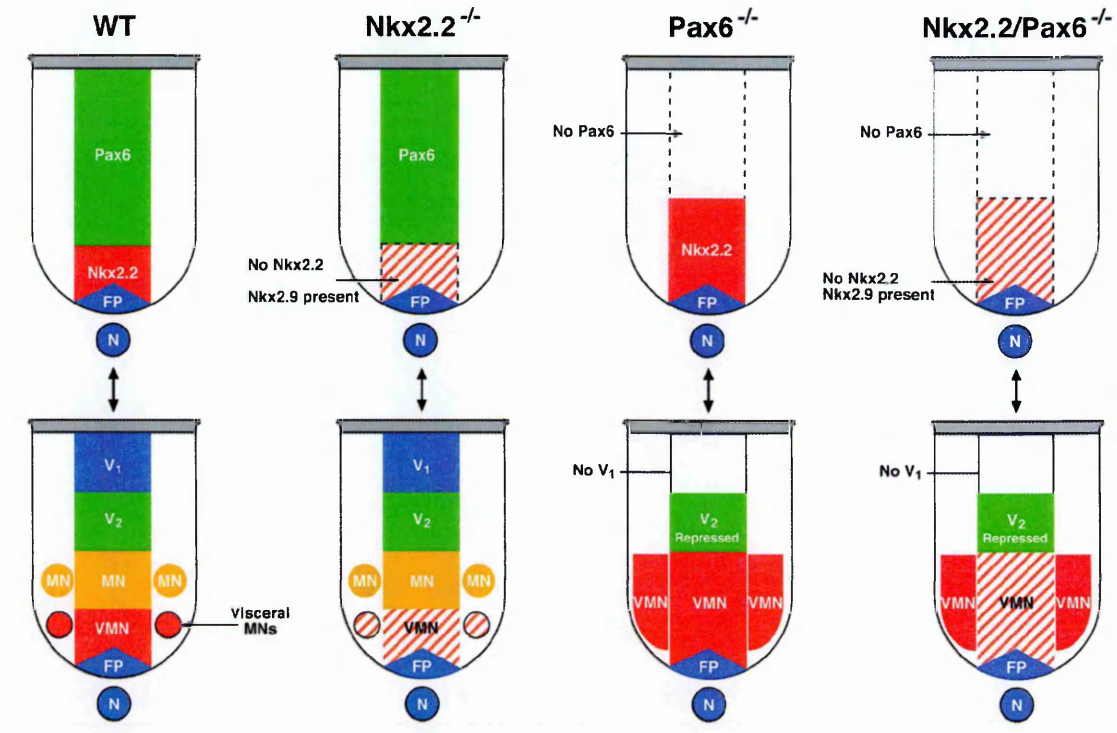

Figure 30 Schematic showing the patterning of the ventral neural tube of the single and double mutants (hindbrain level). [Dorsal (top) – Ventral (bottom)]

5.2 Analysis of the spinal cord phenotype of Nkx2.2/Pax6$^{-/-}$ mice

Initially, we focused our attention on the spinal cord. We first looked at the expression pattern of Nkx2.9, a gene related to Nkx2.2, which is expressed in the same domain as Nkx2.2 and has been previously shown to be almost completely lost in the absence of Nkx2.2 (Briscoe et al.., 1999; Pabst et al.., 1998). Our observations are consistent with those published previously and indicate that Nkx2.9 is expressed at low levels at E10 and is completely extinguished at later stages in Nkx2.2$^{-/-}$ embryos (Fig 32 & 33). Consistent with our predictions this is also true for double mutant embryos (Fig 32 & 33).

To test our hypothesis that the double mutant phenotype would resemble the Nkx2.2$^{-/-}$ phenotype at spinal cord levels we examined the expression of the bHLH gene Ngn3 and the PAS HLH gene Sim1, markers for V3 neurons. In Nkx2.2$^{-/-}$ embryos both Ngn3 and Sim1 are lost, therefore loss of expression of both genes is expected in the Nkx2.2/Pax6$^{-/-}$ embryos.

We first examined Ngn3 expression (Fig 50). In WT embryos at E10.5 Ngn3 is expressed at high levels in V3 neurons and at lower levels in progenitors in the intermediate neural tube. Consistent with previous data, Ngn3 expression in the V3 region is slightly expanded in the Pax6 homozygote mutant embryos, while in Nkx2.2$^{-/-}$ mutants Ngn3 expression is lost in the V3 region. In double mutant embryos expression of Ngn3 in the intermediate neural progenitors appears similar to controls while expression within the V3 domain is lost. This resembles the Nkx2.2 mutant embryos and suggests that V3 neurons are lost in double mutant animals.

To confirm this observation we then examined the expression of Sim1. We first looked at E11.5 WT embryos and compared Sim1 expression pattern with that seen in

mutant embryos. Sim1 is expressed ventrally in WT embryos and marks V3 neurons (Fig 35). As expected, we observed loss of Sim1 in Nkx2.2$^{-/-}$ embryos (Fig 35) and dorsal expansion of Sim1 in Pax6$^{-/-}$ embryos (presumably due to the dorsal expansion of Nkx2.2 (Fig 35) (Ericson et al.., 1997b). In double mutant embryos Sim1 expression was lost in the most ventral region. However, unexpectedly, we observed ectopic Sim1 expression in a more dorsal region in Nkx2.2/Pax6$^{-/-}$ embryos (Fig 35). This position was equivalent to the dorsal region in which ectopic Sim1 expression was observed in the Pax6$^{-/-}$ embryos (Fig 35).

To extend these observations we looked at E10.5 embryos. In WT embryos Sim1 was present ventrally marking the presence of V3 neurons (Fig 34). We consistently observed loss of ventral Sim1 expression in Nkx2.2$^{-/-}$, as expected due to the loss of Nkx2.2 (Fig 34). Moreover, we observed ectopic *Sim1* expression dorsally in Pax6$^{-/-}$ mutants, and in many (<95%) embryos it appears that there are two separate domains, one ventral and one dorsal, that express Sim1. In double mutant embryos we observed a loss of *Sim1* expression in the most ventral regions of the neural tube suggesting that for normal V3 production in the ventral neural tube Nkx2.2 expression is necessary. However, ectopic *Sim1* expression was observed dorsally suggesting that the presence of Pax6 is required for the repression of V3 generation. In the absence of Pax6 in the domain where V2 neurons normally derive, ectopic V3 neurons are generated independent of Nkx2.2. These results suggest that the ectopic expression of Sim1 dorsally in the double mutant embryos is due to the de-repression of *Sim1* consistent with the loss of Pax6.

It was evident that, ectopic Sim1 expression was restricted within what appeared to be the V2 region and not observed throughout the whole Pax6 progenitor domain, for example no ectopic Sim1 was observed in the MN progenitor domain. This suggests the presence of another molecule(s) within the ventral domain responsible for repression of

Sim1. Within somatic MN progenitors, Olig2 is a candidate molecule for restraining Sim1 expression since it would be expected to be maintained within the domain where Sim1 expression was absent. This raises the possibility that Olig2 represses Sim1 induction. To test this idea we examined Olig2 expression in mutant embryos. In WT embryos Olig2 is expressed in the pMN domain (Fig 44 & 45). In contrast, in Pax6 mutants even though Olig2 expression was still present the levels of expression were noticeably decreased (Fig 44). In the Nkx2.2$^{-/-}$ and Nkx2.2/Pax6$^{-/-}$ embryos, ventral expansion of Olig2 expression was observed (Fig 44). This is consistent with the loss of Nkx2.2 and the previously described repressive activity of Nkx2.2 on Olig2 expression (Novitch et al.., 2001). Notably, Olig2 was expressed in the Pax6 and double mutants in the motor neuron region where Sim1 expression was excluded from (Fig 44 & 46). This raises the possibility that in double mutant embryos, Olig2 expression is sufficient to repress V3 neuron generation. Moreover, the ventral expansion of Olig2 in Nkx2.2 mutants raises the possibility that the presence of Olig2 rather than the loss of Nkx2.2 is the reason for the lack of V3 neurons in Nkx2.2$^{-/-}$ embryos. To test this, it will be necessary to make Olig2/Nkx2.2/Pax6 triple mutants that we will further discuss in a later section.

We next turned our attention to V2 neuronal markers Chox10, GATA3 and FoxD3. In WT embryos the markers are expressed dorsally in the MN domain and comprise the domain of V2 neuron generation. Since V3 neurons are generated within the V2 domain in the absence of Pax6 then we might expect downregulation/loss of V2 neuronal markers in Pax6 and double mutants. Consistent with this we observed downregulation of all V2 markers (Fig 37, 38 & 48) in this region of the Pax6$^{-/-}$ and double mutant embryos.

Finally, we turned our attention to somatic motor neurons. Somatic motor neurons derive from progenitor cells within the pMN domain located dorsal to the floorplate. The generation of somatic motor neurons is defined by co-expression of a number of

homeodomain proteins including Isl1/2 and HB9 (Ericson et al.., 1997b; Tanabe et al.., 1998). In the absence of Nkx2.2 there is a ventral expansion of MN and a subsequent loss of V3 interneurons (Fig 29) (Briscoe et al.., 1999). In the absence of Pax6, MN generation is repressed due to the Nkx2.2 expansion dorsally in the pMN domain (Fig 29) (Ericson et al.., 1997b). Based on these observations in double mutant embryos we expected a phenotype similar to that seen in Nkx2.2$^{-/-}$ embryos where somatic motor neuron generation has expanded ventrally within the progenitor domain that gives rise to V3 neurons. Consistent with this, ventral expansion of MN generation in the double mutant was evident by the presence of ectopic MN markers in more ventral positions. In Nkx2.2$^{-/-}$ the expression of Isl1/2, HB9 and Olig2 indicated the presence of motor neurons in the most-ventral progenitor domain (Fig 36, 44 & 46). The same pattern was present in Nkx2.2/Pax6$^{-/-}$ indicating the ectopic generation of motor neurons in more ventral positions within the Nkx2.2 domain that are normally expressed (Fig 36, 44 & 46).

5.3 Analysis of the hindbrain phenotype of Nkx2.2/Pax6$^{-/-}$ mice

We next examined neural patterning and generation of neuronal subtype identities in the hindbrain. Consistent with previously published data (Briscoe et al.., 1999) Nkx2.9 was expressed at high levels in the hindbrain of E10.5 Nkx2.2 mutant embryos (Fig 39). Nkx2.9 expression was also observed in the hindbrain of the Nkx2.2/Pax6 mutants (Fig 39), a result consistent with our predictions for the double mutant phenotype. By E11.5 Nkx2.9 levels were reduced but were still present in the hindbrain of Nkx2.2$^{-/-}$ and Nkx2.2/Pax6$^{-/-}$. Nkx2.2 expression was not present in the hindbrain of Nkx2.2 and double mutants (Fig 31).

In contrast to the spinal cord patterning of Nkx2.2 mutants, where we observe expansion of somatic motor neurons at the expense of V3 neurons in the hindbrain, visceral motor neurons that derive from the ventral most progenitor domain are still present (Fig 40). It has been previously suggested that the functions of Nkx2.9 and Nkx2.2 overlap and the presence of Nkx2.9 in the hindbrain in the $Nkx2.2^{-/-}$ prevents expansion of somatic motor neurons ventrally (Briscoe et al.., 1999).

Consistent with previous studies, we observe a dorsal expansion of visceral motor neurons in Pax6 mutants concomitant with Nkx2.2 expansion dorsally. As a result, in Pax6 mutants a marked reduction in the number of HB9 expressing MNs was observed (Fig 40, 41, 42). These data are consistent with previously published work (Briscoe et al.., 1999).

According to our predictions, $Nkx2.2/Pax6^{-/-}$ mice should show a similar hindbrain phenotype to that of the $Pax6^{-/-}$ mice (Fig 30). Our prediction was that in the absence of Pax6, the presence of Nkx2.9 will result in the repression of somatic MN generation and an expansion in the generation of visceral MNs within the pMN domain. Consistent with this a marked reduction in HB9 and Olig2 expression (somatic motor neuron markers) was evident in double mutant embryos (Fig 42, 43, 45 & 47). Unexpectedly however, dorsal expansion of visceral motor neurons was not observed. The domain of expression of Phox2B appeared to occupy a domain equivalent to wild-type litter mates and did not expand as seen in $Nkx2.2^{-/-}$ (Fig 40). Motor neurons positive for Isl1/2 expression were still present in the double mutant mice (Fig 42) and occupied the region characteristic of visceral MNs.

We next examined the expression of V2 interneuron markers. The number of Chox10 and Gata3 expressing cells appeared slightly increased in double mutant embryos compared to WT (Fig 49) in contrast to our previous observations at spinal cord levels where the opposite effect was observed. These results did not agree with our original

predictions since we expected fewer V2 neurons similar to the phenotype observed in Pax6-/- embryos. Taking into account the lack of expansion of vMN in double mutant embryos a possible explanation could be that V2 neurons are now generated ventrally in the domain that would normally be expected to produce somatic motor neurons.

Figure 31: Nkx2.2 expression is present in the ventral most region of the spinal cord and hindbrain of E 10.5 WT embryos (A & E) (using immunohistochemistry). In single Nkx2.2$^{-/-}$ (B & F) and double Nkx2.2/Pax6$^{-/-}$ (D & H) mutants no Nkx2.2 expression is observed while in Pax6$^{-/-}$ mutants Nkx2.2 expands dorsally (C & G) (using immunohistochemistry). Pax 6 expression is present on the dorsal half of the ventral neural tube of WT and Nkx2.2 (A, B, E & F) mutants but absent from Pax6 and double mutant embryos (C, D, G & H) . All spinal cord sections are from the forelimb region of the embryo. All hindbrain sections are from the caudal hindbrain region.

Figure 32: *Nkx2.9* expression is normal in the spinal cord of Wt (A & a) and Pax6$^{-/-}$ (B & b) mutants while almost extinguished in the Nkx2.2$^{-/-}$ (C & c) and Nkx2.2/Pax6$^{-/-}$ (D & d) mutants (using *in situ* hybridisation). All spinal cord sections are from the forelimb region of the embryo. [a-d are higher magnifications (x 20) of the upper panels (x10)]

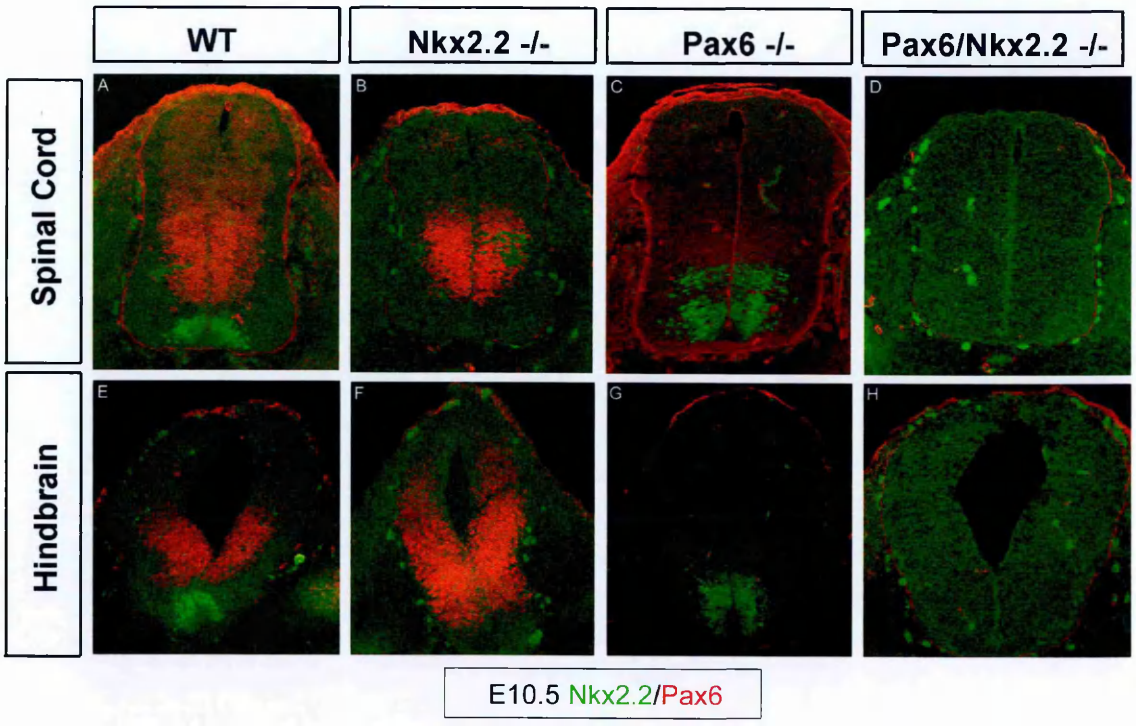

Figure 31 Spinal cord and hindbrain expression of Nkx2.2 & Pax6 in WT, single and double mutants [Dorsal (top) – Ventral (bottom)].

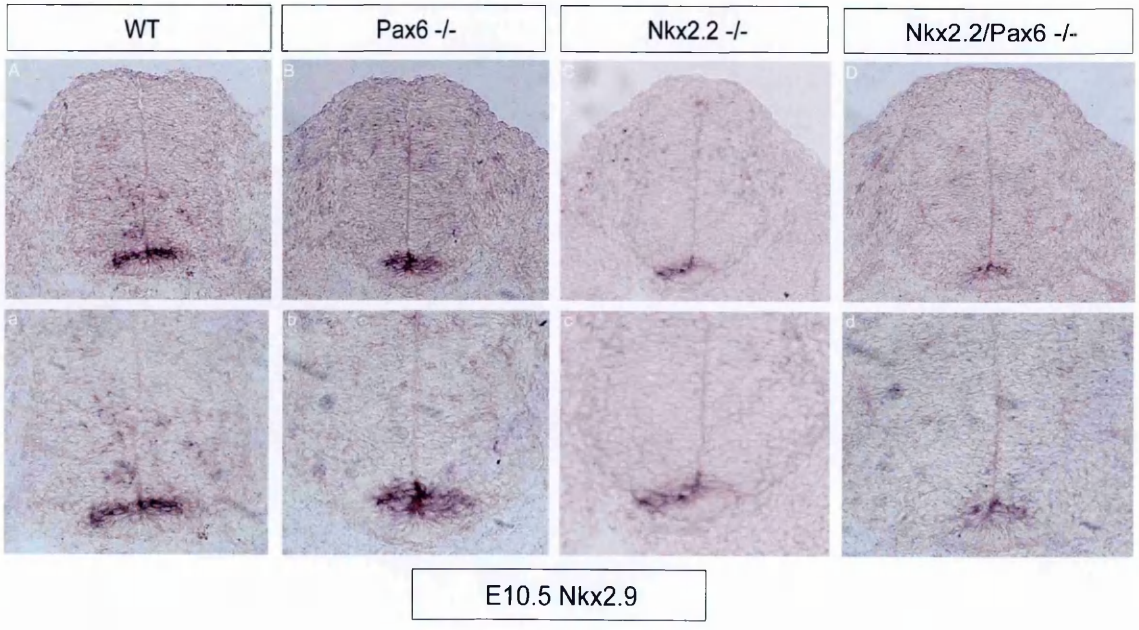

Figure 32 Spinal cord expression of *Nkx2.9* in E10.5 WT, single and double mutants [Dorsal (top) – Ventral (bottom)].

Figure 33: *Nkx2.9* expression is extinguished from the spinal cord of E 11.5 WT (A), Pax6$^{-/-}$ (B), Nkx2.2$^{-/-}$ (C) and Nkx2.2/Pax6$^{-/-}$ (D) mutants (using *in situ* hybridisation). All spinal cord sections are from the forelimb region of the embryo.

Figure 34: In E 10.5 WT embryos Sim1 is present ventrally marking the presence of V3 neurons (A & a) (using *in situ* hybridisation). *Sim1* expression in Nkx2.2$^{-/-}$ is lost since Nkx2.2 activity is required for the generation of V3 neurons (C & c) (using *in situ* hybridisation). Ectopic *Sim1* expression is evident dorsally in Pax6$^{-/-}$ mutants, indicating the presence of two separate domains, one ventral and one dorsal that express Sim1 (B & b) (using *in situ* hybridisation). In double mutant embryos loss of *Sim1* expression in the most ventral regions of the neural tube suggests that for normal V3 production in the ventral neural tube Nkx2.2 expression is necessary (D & d) (using *in situ* hybridisation). Ectopic *Sim1* expression dorsally suggests that the presence of Pax6 is required for the repression of V3 generation (D & d) (using *in situ* hybridisation). All spinal cord sections are from the forelimb region of the embryo. [a-d are higher magnifications (x 20) of the upper panels (x10)]

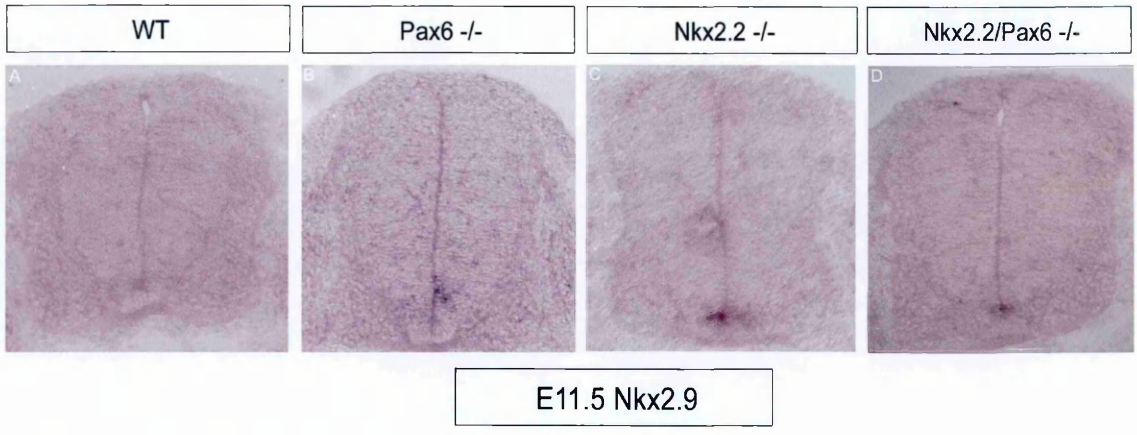

Figure 33 Spinal cord expression of *Nkx2.9* in E11.5 WT, single and double mutants [Dorsal (top) – Ventral (bottom)].

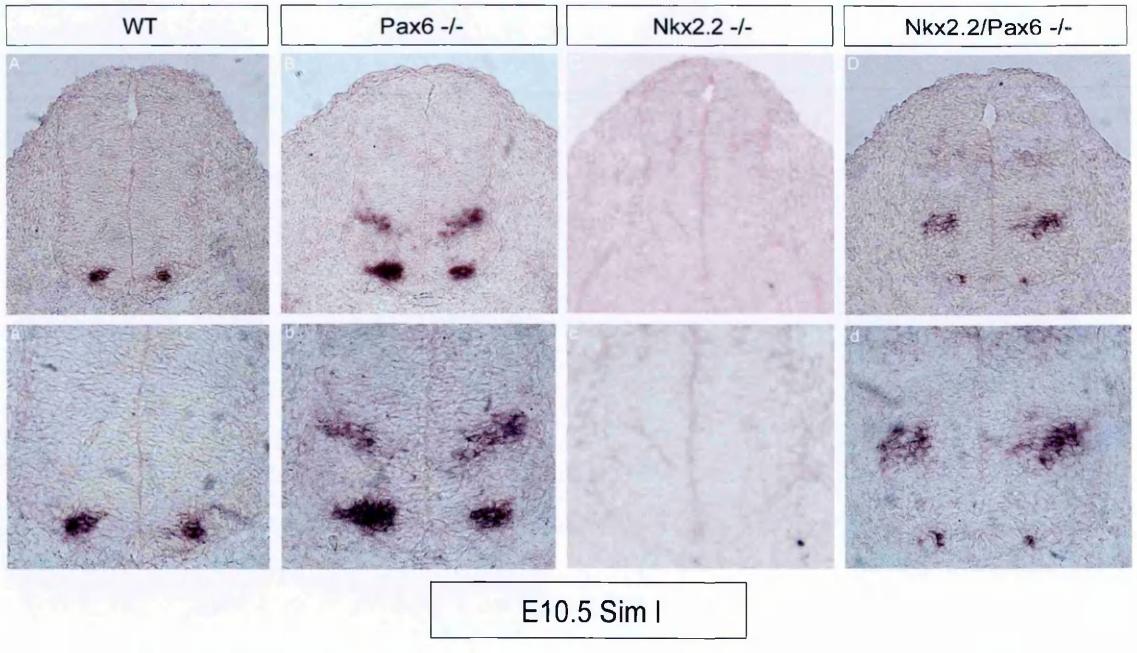

Figure 34 Spinal cord expression of *Sim1* in E10.5 WT, single and double mutants [Dorsal (top) – Ventral (bottom)].

Figure 35: *Sim1* expression ventrally in E11.5 WT embryos marking V3 neurons (A & a) (using *in situ* hybridisation). *Sim1* expression is lost in Nkx2.2$^{-/-}$ embryos (C & c) and dorsally expanded in Pax6$^{-/-}$ embryos (B & b) rather than expressed in a ventral and a dorsal region as previously seen in E 10.5 embryos (Fig 34 B & b) (using *in situ* hybridisation). In double mutant embryos *Sim1* expression was lost in the most ventral region but was present dorsally (D & d) (using *in situ* hybridisation). All spinal cord sections are from the forelimb region of the embryo. [a-d are higher magnifications (x 20) of the upper panels (x10)]

Figure 36: MN generation is restricted to the pMN domain of the spinal cord of WT (A & a) and Pax6$^{-/-}$ (B & b) and is absent from the ventral most region of the neural tube (using immunohistochemistry). Ventral expansion of MN generation in the Nkx2.2$^{-/-}$ (C & c) and double mutant animals (D & d) is evident by the presence of ectopic MN markers Isl1/2 and HB9 in more ventral positions (using immunohistochemistry). All spinal cord sections are from the forelimb region of the embryo. [a-d are higher magnifications (x 20) of the upper panels (x10)]

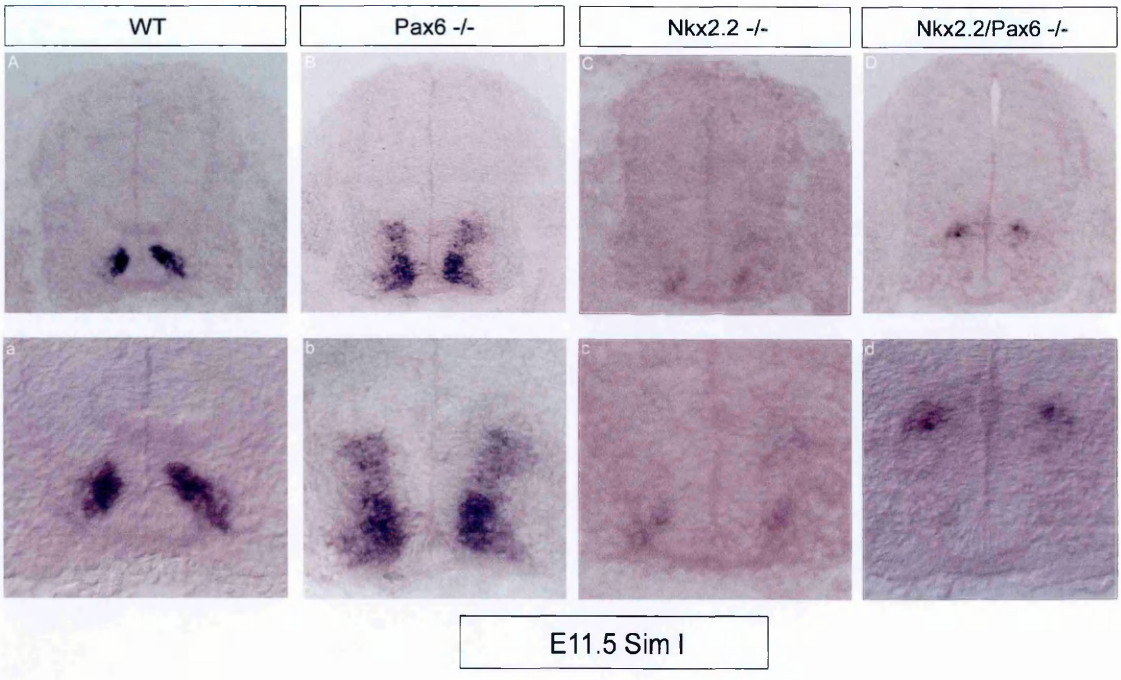

Figure 35 Spinal cord expression of *Sim1* in E11.5 WT, single and double mutants [Dorsal (top) – Ventral (bottom)].

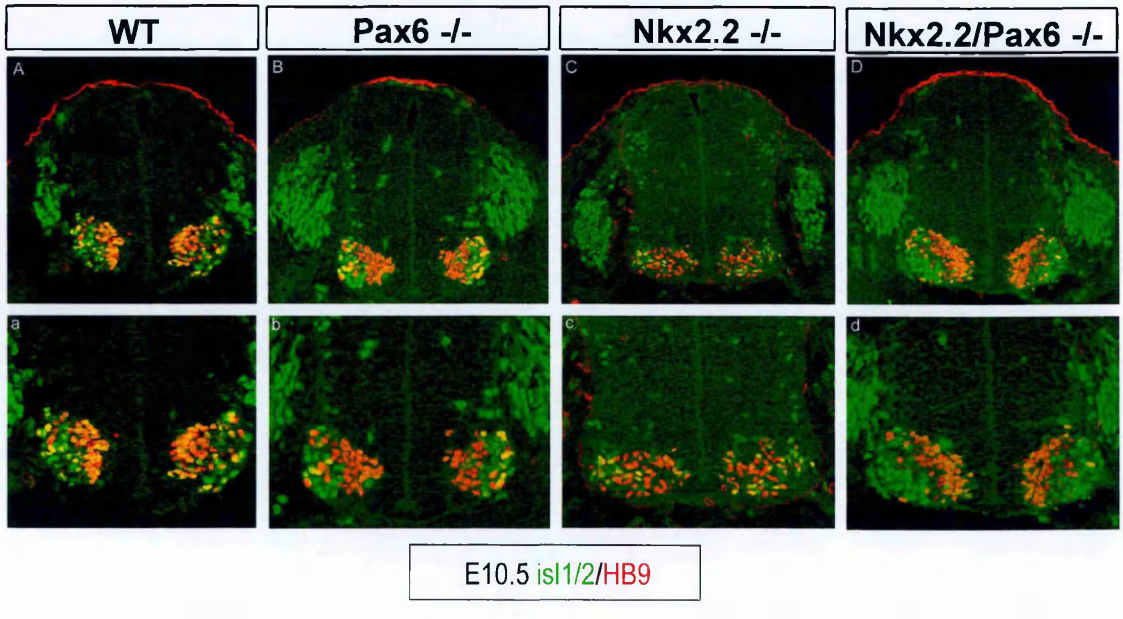

Figure 36 Spinal cord expression of Islet1/2 & HB9 in E10.5 WT, single and double mutants [Dorsal (top) – Ventral (bottom)].

Figure 37: In the spinal cord of E11.5 WT (A & a) and Nkx2.2$^{-/-}$ (C & c) embryos *FoxD3* expression is present dorsally to the MN domain and comprises the domain of V2 neuron generation (using *in situ* hybridisation). Downregulation of *FoxD3* expression in Pax6$^{-/-}$ (B & b) and double mutant embryos (D & d) is due to generation of V3 neurons within the V2 domain in the absence of Pax6 (using *in situ* hybridisation). All spinal cord sections are from the forelimb region of the embryo. [a-d are higher magnifications (x 20) of the upper panels (x10)]

Figure 38: *FoxD3* expression at earlier stages (E10.5) is consistent with our observations at E11.5 (Fig 37) (using *in situ* hybridisation). All spinal cord sections are from the forelimb region of the embryo. [a-d are higher magnifications (x 20) of the upper panels (x10)]

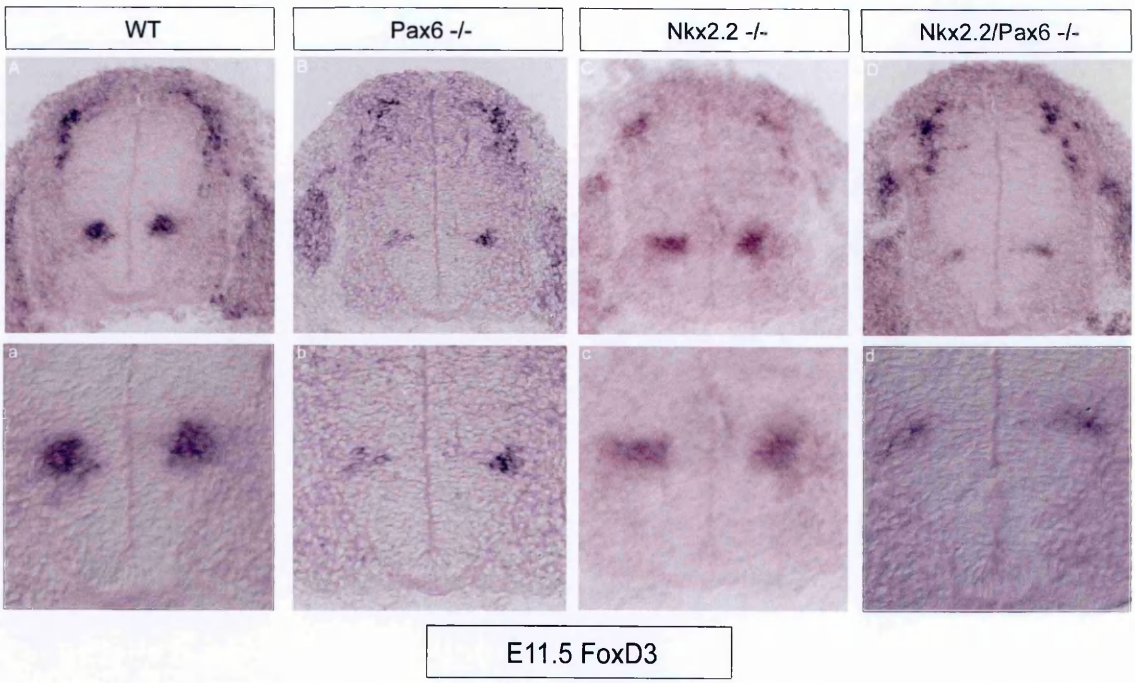

Figure 37 Spinal cord expression of FoxD3 in E11.5 WT, single and double mutants [Dorsal (top) – Ventral (bottom)].

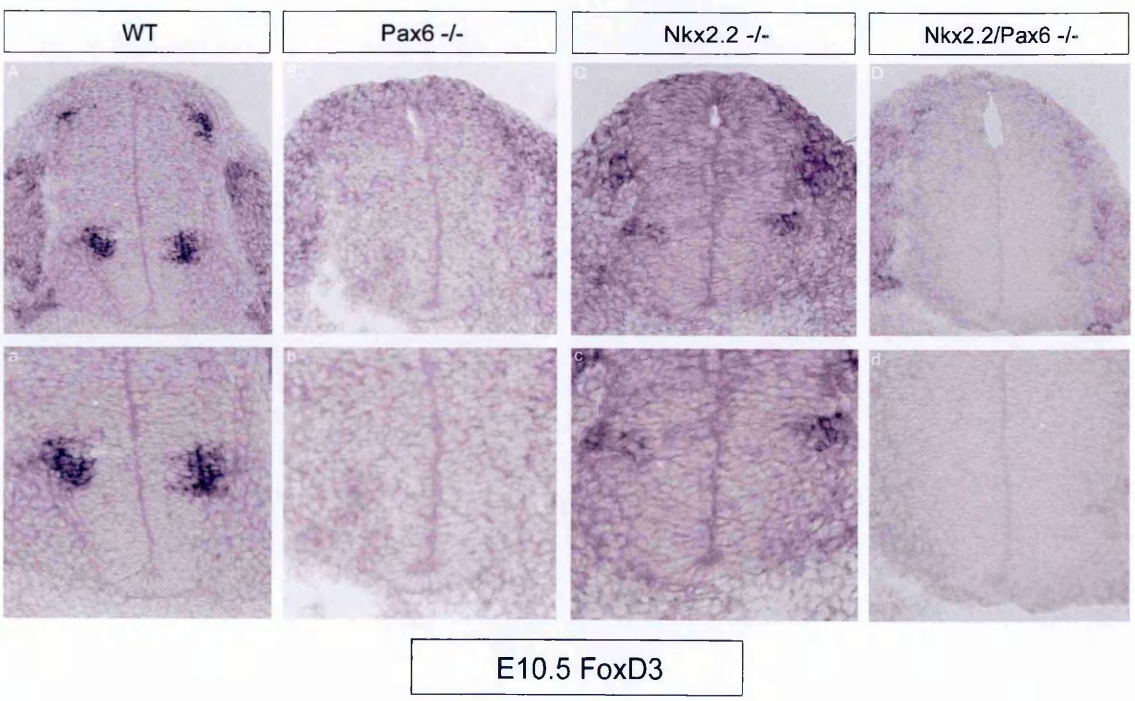

Figure 38 Spinal cord expression of FoxD3 in E10.5 WT, single and double mutants [Dorsal (top) – Ventral (bottom)].

Figure 39: *Nkx2.9* expression is normal in the hindbrain of Wt (A & a) and $Pax6^{-/-}$ (B & b) mutants (using *in situ* hybridisation). In contrast to the spinal cord level *Nkx2.9* expression is also present in the hindbrain of $Nkx2.2^{-/-}$ (C & c) and $Nkx2.2/Pax6^{-/-}$ (D & d) mutants. (using *in situ* hybridisation). All hindbrain sections are from the caudal hindbrain region. [a-d are higher magnifications (x 20) of the upper panels (x10)]

Figure 40: Phox2B expression in the hindbrain of $Nkx2.2^{-/-}$ shows that visceral motor neurons that derive from the ventral most progenitor domain are still present (C & c) (using immunohistochemistry). Visceral motor neurons expand dorsally in Pax6 mutants concomitant with Nkx2.2 expansion dorsally resulting in a marked reduction in the number of HB9 expressing MNs (B & b) (using immunohistochemistry). In double mutants (D & d) no dorsal expansion of visceral motor neurons was observed, as seen in $Nkx2.2^{-/-}$ (C & c), and the domain of expression of Phox2B appeared to occupy a domain equivalent to wild-type litter mates (A & a) (using immunohistochemistry). All hindbrain sections are from the caudal hindbrain region. [a-d are higher magnifications (x 20) of the upper panels (x10)]

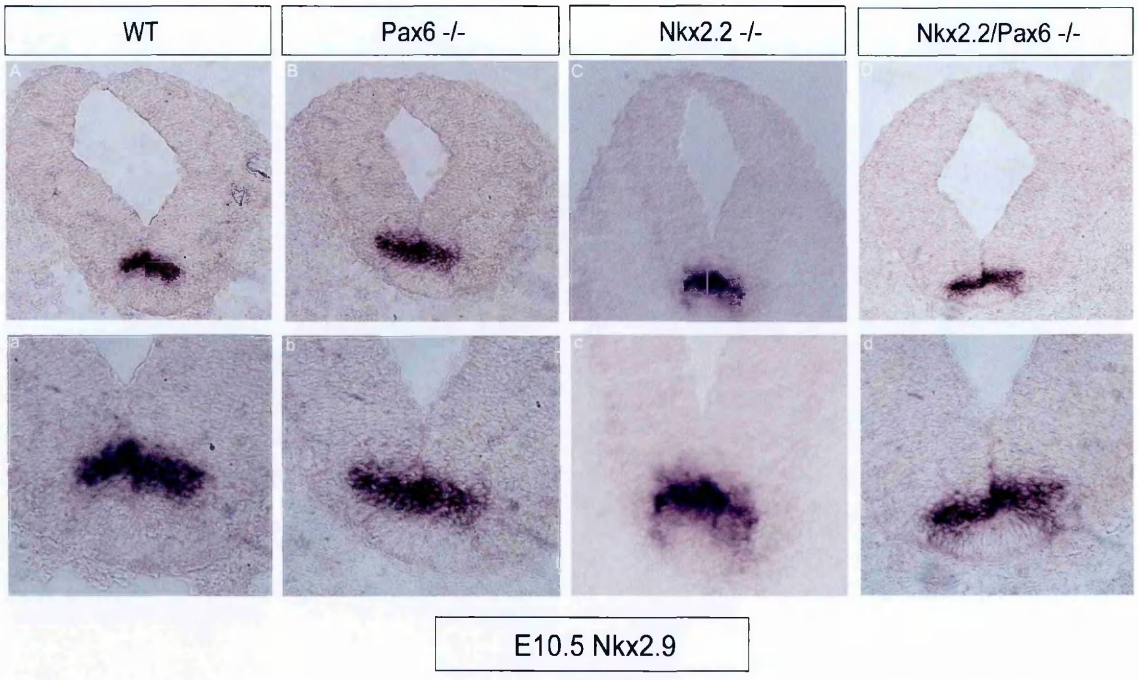

Figure 39 Hindbrain expression of Nkx2.9 in E10.5 WT, single and double mutants [Dorsal (top) – Ventral (bottom)].

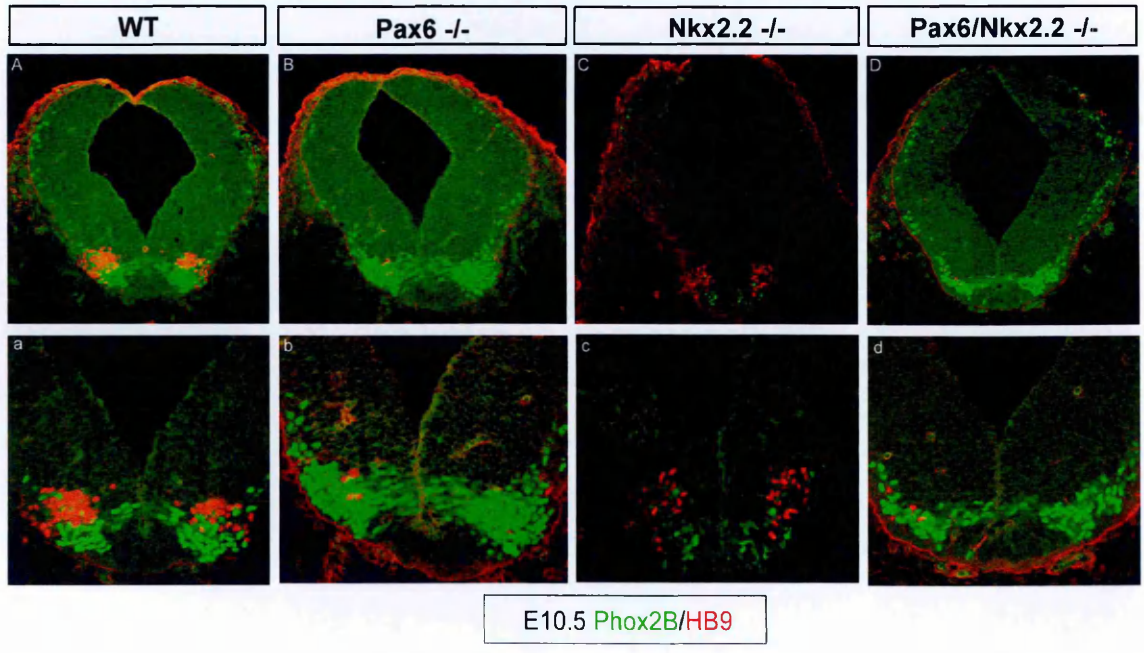

Figure 40 Hindbrain expression of Phox2B & HB9 in E10.5 WT, single and double mutants [Dorsal (top) – Ventral (bottom)].

Figure 41: Nkx2.2 expansion dorsally in the hindbrain of Pax6 mutants and the presence of Nkx2.9 in the hindbrain of the double mutants results in the loss of HB9 expressing MNs (B, b, D & d) (using immunohistochemistry). HB9 expressing MNs are present in WT (A & a) and Nkx2.2 mutants (C & c) (using immunohistochemistry).. All hindbrain sections are from the caudal hindbrain region. [a-d are higher magnifications (x 20) of the upper panels (x10)]

Figure 42: Motor neurons positive for Isl1/2 expression are present in the hindbrain of double (D & d) and Pax6 (B & b) mutant mice occupying the region characteristic of visceral MNs (using immunohistochemistry). All hindbrain sections are from the caudal hindbrain region. [a-d are higher magnifications (x 20) of the upper panels (x10)]

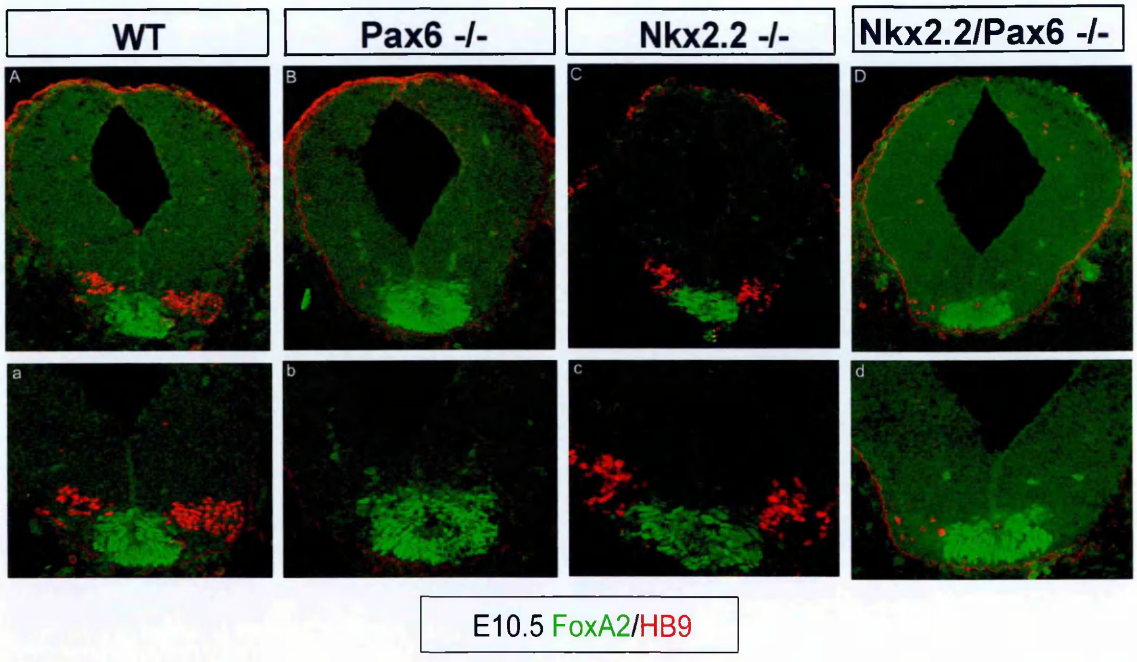

Figure 41 Hindbrain expression of FoxA2 & HB9 in E10.5 WT, single and double mutants [Dorsal (top) – Ventral (bottom)].

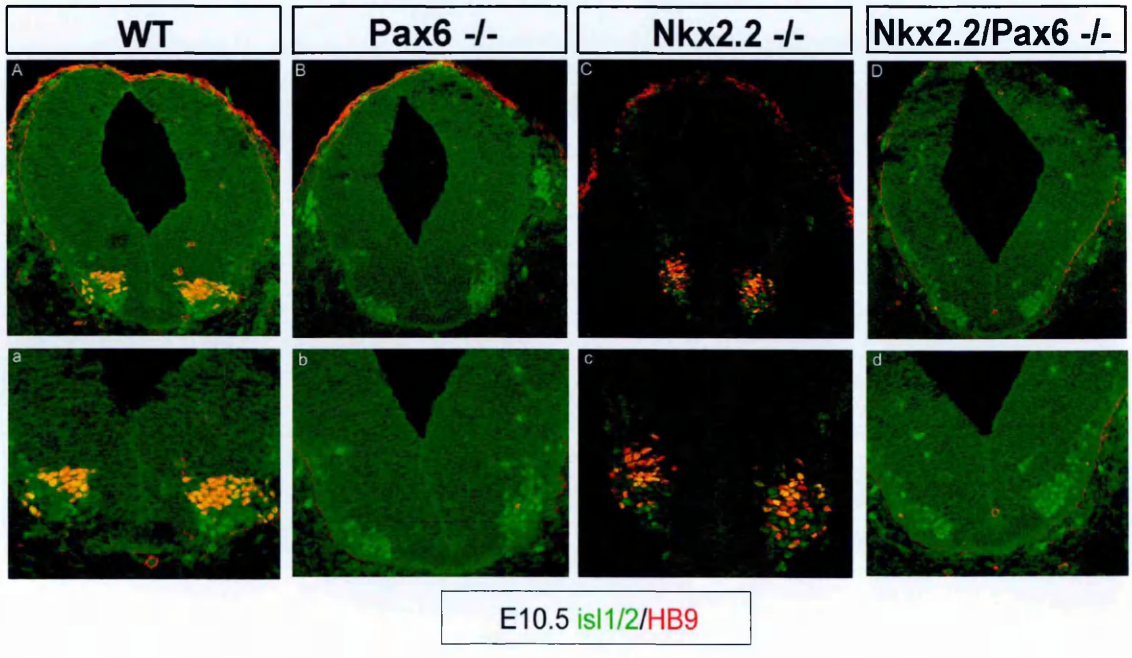

Figure 42 Hindbrain expression of Islet1/2 & HB9 in E10.5 WT, single and double mutants [Dorsal (top) – Ventral (bottom)].

Figure 43: Nkx2.2 expansion dorsally in the hindbrain of Pax6 mutants and the presence of Nkx2.9in the hindbrain of the double mutants results in the loss of Olig2 expressing MNs (B, b, D & d) (using immunohistochemistry). Olig2 expressing MNs are present in WT (A & a) and Nkx2.2 mutants (C & c) (using immunohistochemistry). All hindbrain sections are from the caudal hindbrain region. [a-d are higher magnifications (x 20) of the upper panels (x10)]

Figure 44: In the spinal cord of WT embryos Olig2 is expressed in the pMN domain (A & a) (using immunohistochemistry). In Pax6 mutants Olig2 expression is still present but the levels of expression are decreased (B & b) (using immunohistochemistry). In the Nkx2.2$^{-/-}$ and Nkx2.2/Pax6$^{-/-}$ embryos, ventral expansion of Olig2 expression is observed (c & d) (using immunohistochemistry). All spinal cord sections are from the forelimb region of the embryo. [a-d are higher magnifications (x 20) of the upper panels (x10)]

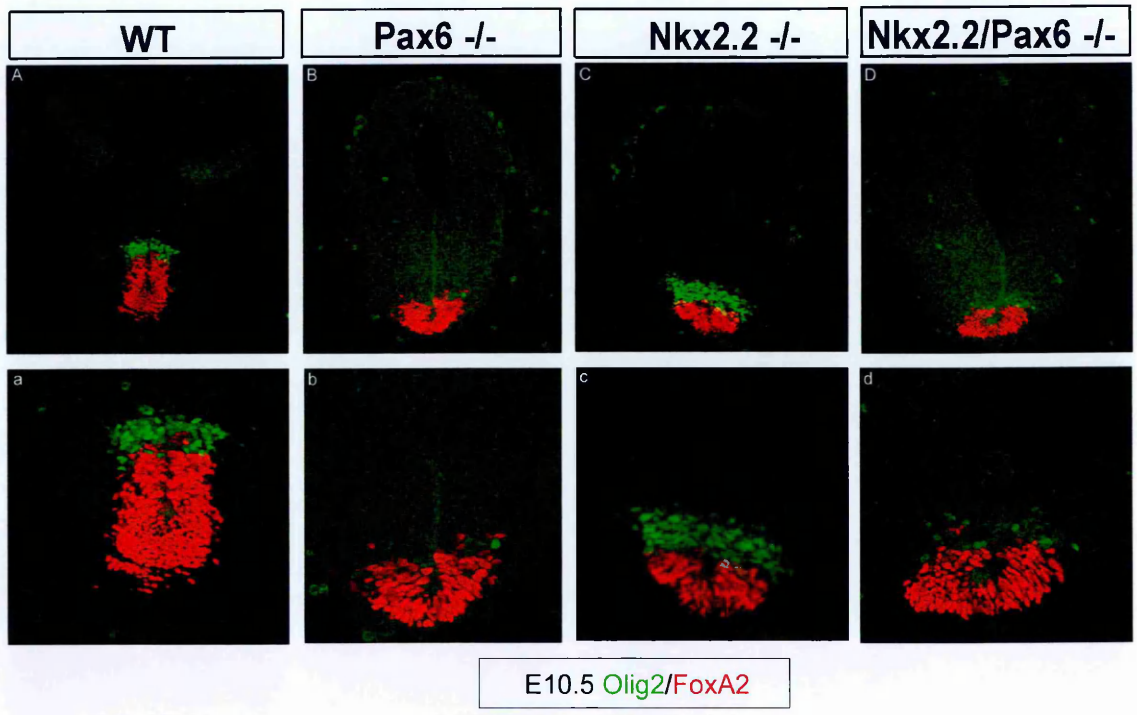

Figure 43 Hindbrain expression of Olig2 & FoxA2 in E10.5 WT, single and double mutants [Dorsal (top) – Ventral (bottom)].

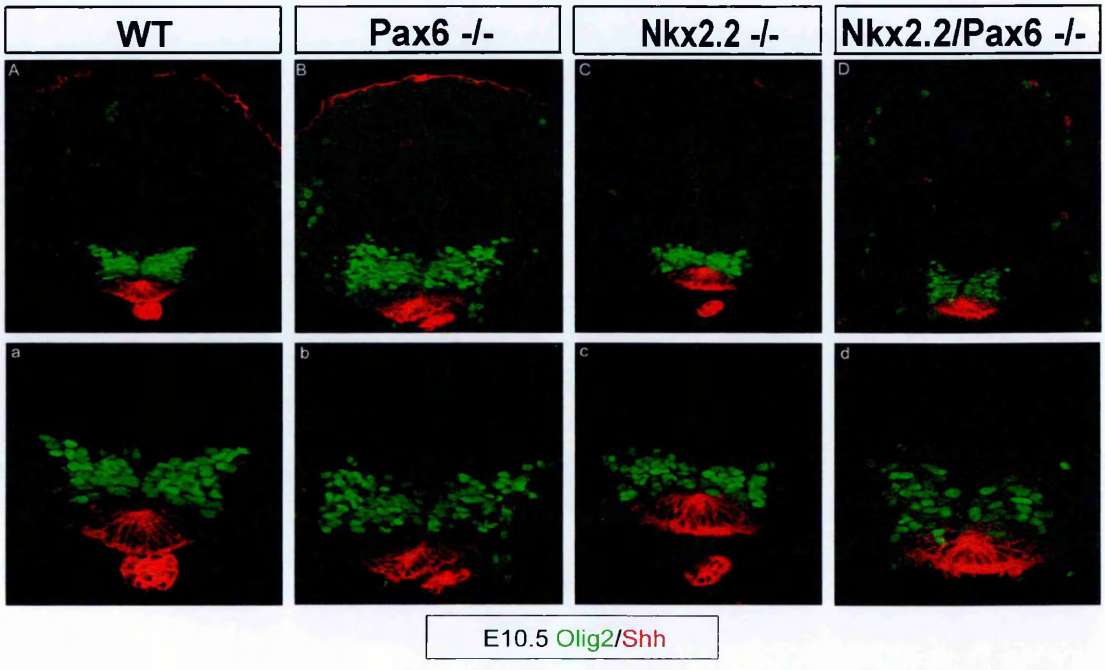

Figure 44 Spinal cord expression of Olig2 & Shh in E10.5 WT, single and double mutants [Dorsal (top) – Ventral (bottom)].

Figure 45: Nkx2.2 expansion dorsally in the hindbrain of Pax6 mutants and the presence of Nkx2.9 in the hindbrain of the double mutants results in the loss of Olig2 expressing MNs (B, b, D & d) (using immunohistochemistry). Olig2 expressing MNs are present in WT (A & a) and Nkx2.2 mutants (C & c) (using immunohistochemistry). All hindbrain sections are from the caudal hindbrain region. [a-d are higher magnifications (x 20) of the upper panels (x10)]

Figure 46: In the spinal cord of Nkx2.2$^{-/-}$ and Nkx2.2/Pax6$^{-/-}$ embryos, ventral expansion of Olig2 expression is observed (c & d) in the region where Nkx2.2 is normally expressed (using immunohistochemistry). All spinal cord sections are from the forelimb region of the embryo. [a-d are higher magnifications (x 20) of the upper panels (x10)]

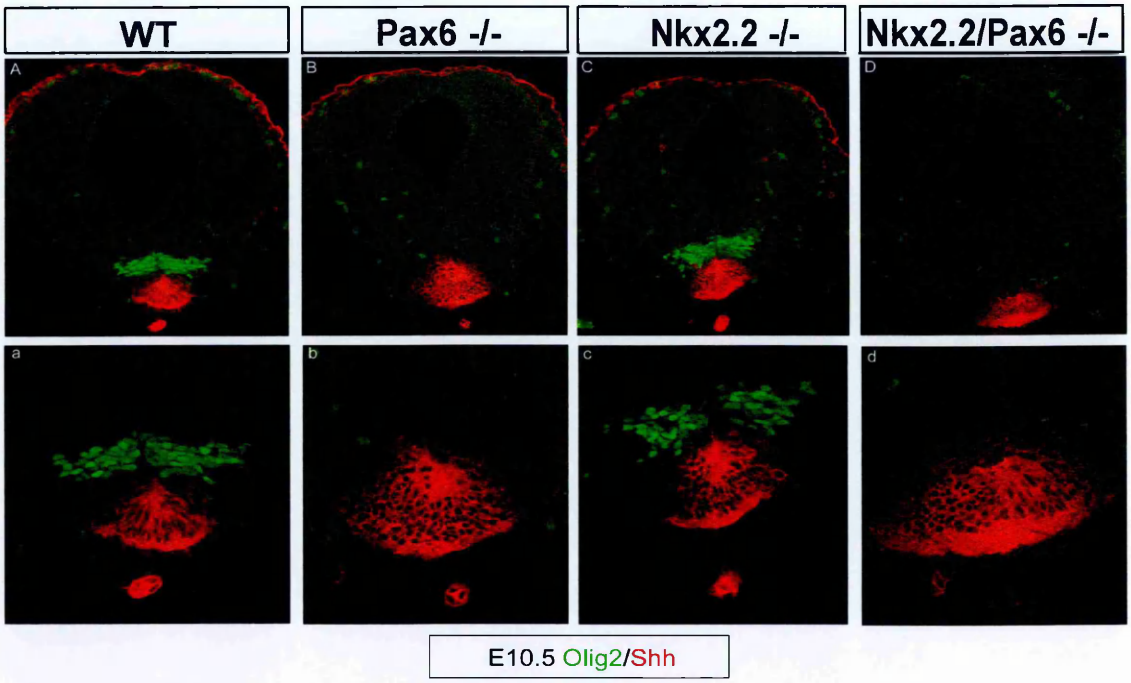

Figure 45 Hindbrain expression of Olig2 & Shh in E10.5 WT, single and double mutants [Dorsal (top) – Ventral (bottom)].

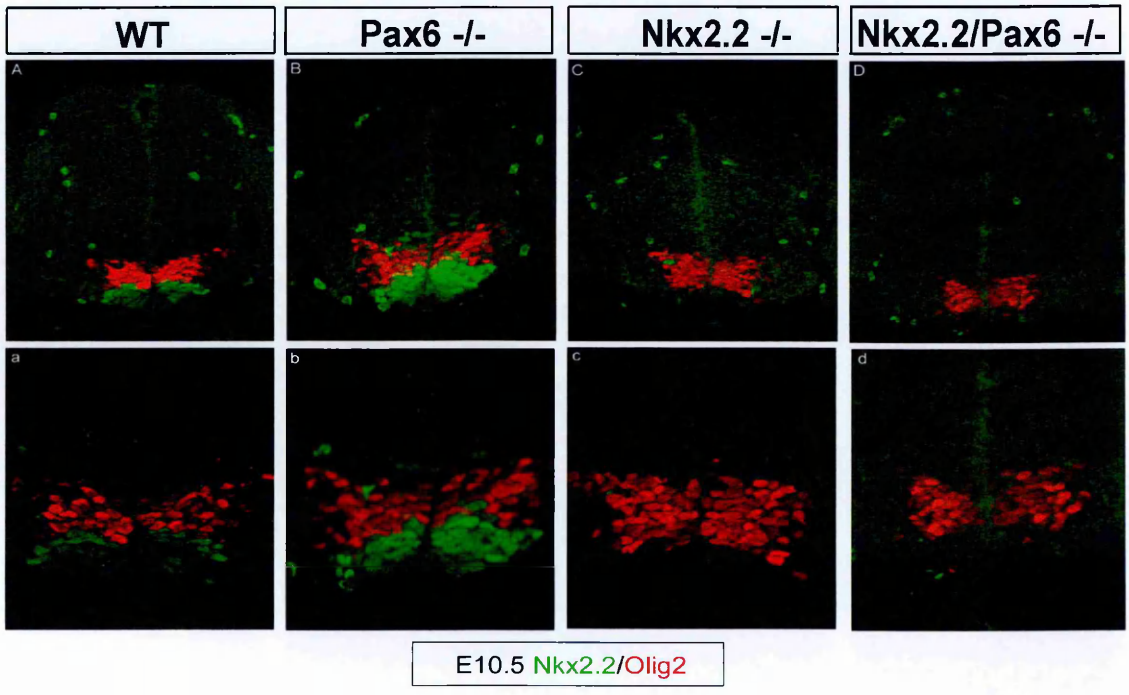

Figure 46 Spinal cord expression of Nkx2.2 & Olig2 in E10.5 WT, single and double mutants [Dorsal (top) – Ventral (bottom)].

Figure 47: Nkx2.2 expansion dorsally in the hindbrain of Pax6 mutants and the presence of Nkx2.9in the hindbrain of the double mutants results in the downregulation/loss of Olig2 expressing MNs (B, b, D & d) (using immunohistochemistry). Olig2 expressing MNs are present in WT (A & a) and Nkx2.2 mutants (C & c) (using immunohistochemistry). All hindbrain sections are from the caudal hindbrain region. [a-d are higher magnifications (x 20) of the upper panels (x10)]

Figure 48: In the spinal cord of E10.5 WT (A & a) and Nkx2.2$^{-/-}$ (C & c) embryos V2 interneuron markers Chox10 and Gata3 are expressed normally (using immunohistochemistry). Downregulation of both markers in Pax6$^{-/-}$ (B & b) and double mutant embryos (D & d) is due to generation of V3 neurons within the V2 domain in the absence of Pax6 (using immunohistochemistry). All spinal cord sections are from the forelimb region of the embryo. [a-d are higher magnifications (x 20) of the upper panels (x10)]

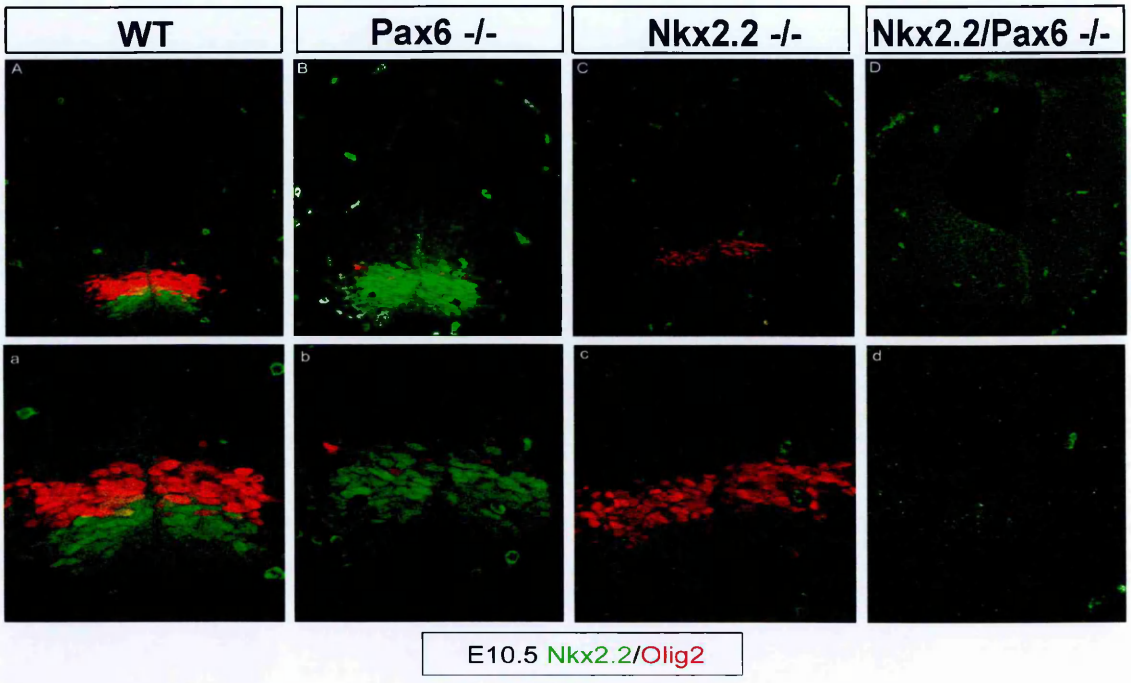

Figure 47 Hindbrain expression of Nkx2.2 & Olig2 in E10.5 WT, single and double mutants [Dorsal (top) – Ventral (bottom)].

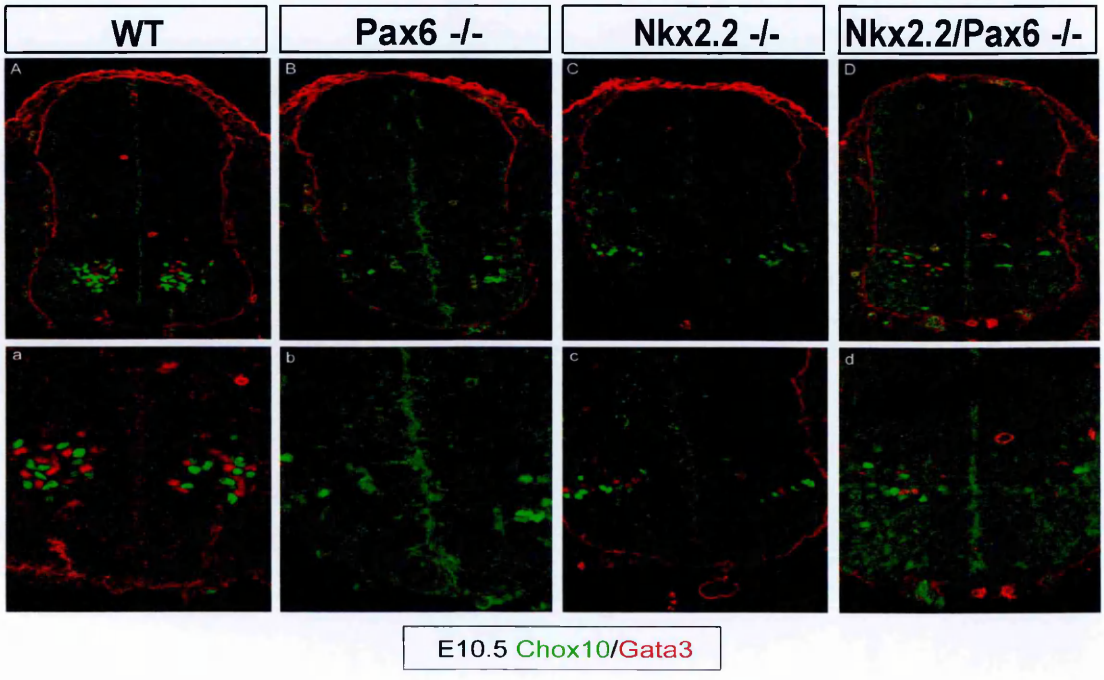

Figure 48 Spinal cord expression of Chox10 & Gata3 in E10.5 WT, single and double mutants [Dorsal (top) – Ventral (bottom)].

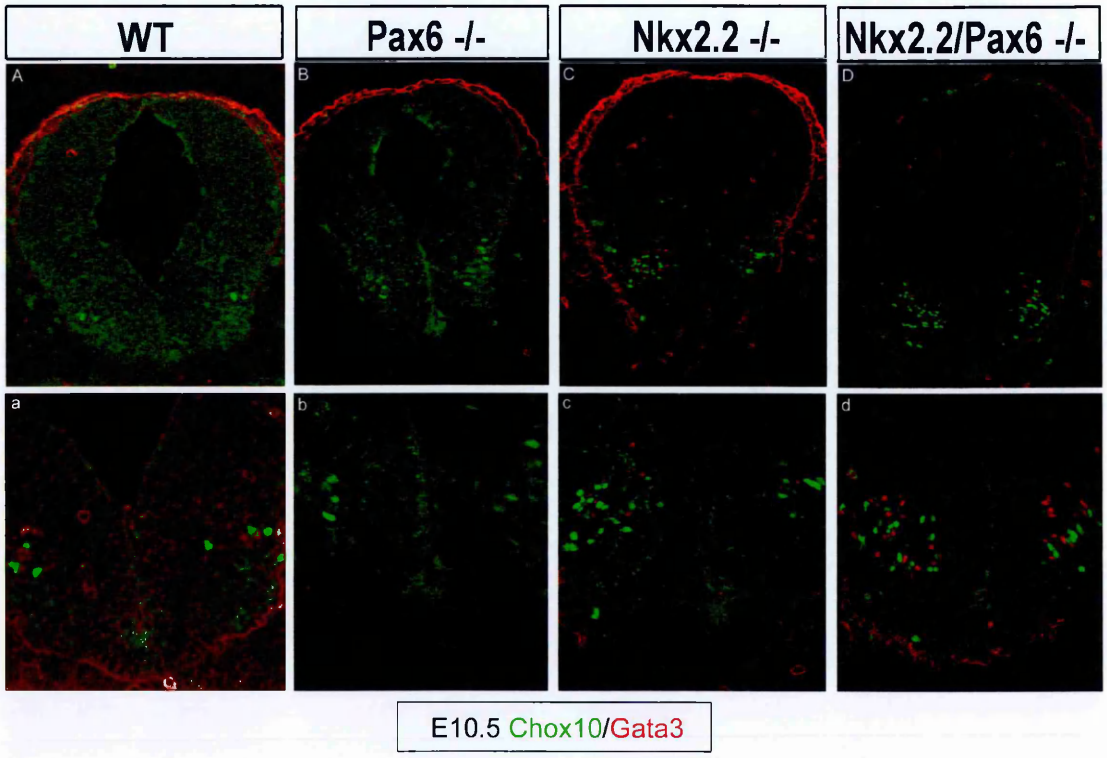

Figure 49 Hindbrain expression of Chox10 & Gata3 in E10.5 WT, single and double mutants [Dorsal (top) – Ventral (bottom)].

The number of Chox10 and Gata3 expressing cells appeared slightly increased in double mutant embryos (d) compared to WT (a) in contrast to our previous observations at spinal cord levels where the opposite effect was observed (Fig 48) (using immunohistochemistry). All hindbrain sections are from the caudal hindbrain region. [a-d are higher magnifications (x 20) of the upper panels (x10)]

6. Results

6.1 Analysis of the neural patterning in the spinal cord the $Ngn3^{\Delta/-}$ mutant mice and correlation of the $Ngn3^{\Delta/-}$ phenotype with that of $Nkx2.2^{-/-}$ and $Nkx2.2/Pax6^{-/-}$ mice.

Neurogenins belong to the family of bHLH transcription factors. They define distinct progenitor populations in the developing CNS and represent markers of the origins of neuronal diversity (Sommer et al.., 1996). Ngn3 expression in the spinal cord has been shown to coincide with early neurogenesis and gliogenesis (Lee et al.., 2003; Sommer et al.., 1996). However, the role and function of Ngn3 in the spinal cord during embryonic development has not been clearly resolved.

Previous studies have shown that Ngn3 is expressed in the ventral neural tube in the same domain as Nkx2.2 expression (Sommer et al.., 1996). Moreover, in $Nkx2.2^{-/-}$ mutant mice there is a loss of Ngn3 expression as well as Sim1 expression (Briscoe et al.., 1999) (Fig 50 & 34). This raises the possibility that Ngn3 may be a downstream mediator of Nkx2.2. During the course of these studies an analysis of the spinal cord phenotype of mice harbouring a null mutation in Ngn3 was reported (Lee et al.., 2003). These data indicated that loss of Ngn3 resulted in a disruption/loss of Nkx2.2 expression (Lee et al.., 2003) and a decrease in the expression of the V3 interneuron marker, *Sim1*, was also observed (Lee et al.., 2003). However, the generation of motor neurons, identified by *Islet1/2* expression, was reportedly normal and there was no indication that motor neuron generation expanded

ventrally as previously observed in the Nkx2.2$^{-/-}$ mice (Briscoe et al.., 1999; Lee et al.., 2003).

Based on this study it seemed possible that expression of Ngn3 and Nkx2.2 could be interdependent and that both genes are required for correct neuronal patterning of the most ventral regions of the neural tube. However, the majority of the analysis reported by Lee et al. (Lee et al.., 2003) was carried out in embryos at E11.5 or older. This is a later time point than used in the majority of our studies. The role of Ngn3 in the early development of the V3 domain therefore remained unclear. We considered three possibilities. First, that Nkx2.2 is required for Ngn3 expression which in turn is required for induction of *Sim1*. Second, that Ngn3 is required for Nkx2.2 expression which then induces *Sim1*. Third, that Ngn3 and Nkx2.2 expression depend on each other and both are required for normal Sim1 induction.

To distinguish between these models we first analysed the expression of Ngn3 using *in situ* hybridisation in E10.5 embryos. Consistent with previously published data Ngn3 was expressed in cells adjacent to the floorplate in the same domain as Nkx2.2 (Fig 50). Additionally, Ngn3 expression was lost in the Nkx2.2$^{-/-}$ mice (Fig 50). Furthermore, we also observed loss of Ngn3 expression in the Nkx2.2/Pax6$^{-/-}$ mice (Fig 50). Taken together these data indicate that Nkx2.2 is required for Ngn3 induction in V3 domain.

Next, we examined ventral neural tube patterning and the generation of V3 neurons in Ngn3 mutant animals. As previously demonstrated the expression of the V3 neuronal marker, *Sim1*, was lost in the Nkx2.2$^{-/-}$ and severely downregulated in the Nkx2.2/Pax6$^{-/-}$ mutant embryos (Fig 51). However, in contrast to the published data of Lee et al. (Lee et al.., 2003), the expression of *Sim1* appeared normal in Ngn3$^{-/-}$ at E10.5 and E11.5 (Fig 51 & 54). These data suggest that Ngn3 is not directly required for the induction of Sim1. However, it is possible that Ngn3 may be required for the continued expression of Sim1 or the generation of the normal numbers of V3 neurons.

We examined and compared the expression of Nkx2.2 in WT and Ngn3 mutant embryos. Nkx2.2 expression at E11.5 also appeared normal (Fig 52) in contrast to previously published data (Lee et al.., 2003) that showed Nkx2.2 expression to be less in the knockout than in the control. To further assess ventral neural tube development in Ngn3 mutants we examined the expression pattern of the MN marker HB9 and the V2 neuron markers Chox10 and Gata3. HB9 expression was unaffected in Ngn3 mutant embryos when compared to wild type embryos (Fig 53). This indicates that Ngn3 is not required to define the ventral limit of MN generation. Similarly, expression of V2 neuronal markers, Chox10 and Gata3, was not affected in Ngn3 mutants when compared with WT embryos (Fig53 & 52). At E11.5 the generation of V2 neurons appears normal and does not seem to be affected by the loss of Ngn3 (Fig 53 & 52).

Taken together these data indicate that Nkx2.2 acts upstream of Ngn3 and is required for Ngn3 expression. Moreover Ngn3 does not appear to be required for induction of V3 neurons or to determine the ventral limit of MN or V2 neuron generation.

Figure 50: *Ngn3* (using *in situ* hybridisation) expression is present ventrally, adjacent to the floor plate in the same domain as Nkx2.9 expression, in the spinal cord of Wt (A & a) and $Pax6^{-/-}$ (B & b) mutants but absent from the ventral spinal cord region of the $Nkx2.2^{-/-}$ (C & c) and $Nkx2.2/Pax6^{-/-}$ (D & d) mutants. All spinal cord sections are from the forelimb region of the embryo. [a-d are higher magnifications (x 20) of the upper panels (x10)]

Figure 51: The V3 neuronal marker, *Sim1*, is lost in the $Nkx2.2^{-/-}$ (C & c) and severely downregulated in the $Nkx2.2/Pax6^{-/-}$ mutant embryos (D & d) (using *in situ* hybridisation). The expression of *Sim1* appears normal in $Ngn3^{-/-}$ mutants (B & b). All spinal cord sections are from the forelimb region of the embryo. [a-d are higher magnifications (x 20) of the upper panels (x10)]

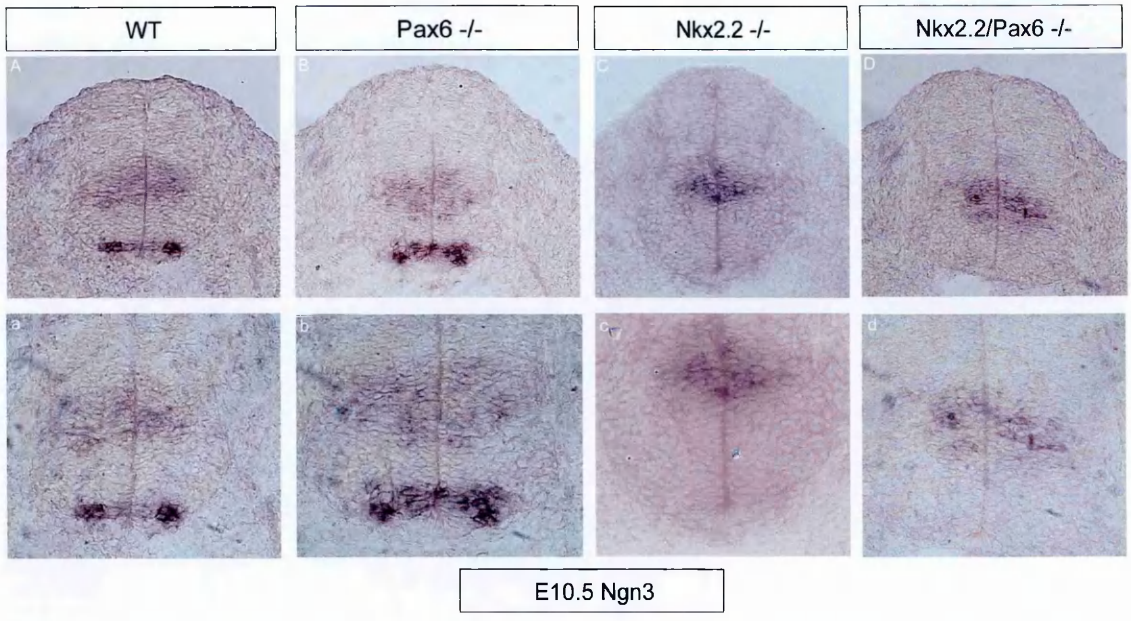

Figure 50 Spinal cord expression of *Ngn3* in E10.5 WT, single and double mutants [Dorsal (top) – Ventral (bottom)]

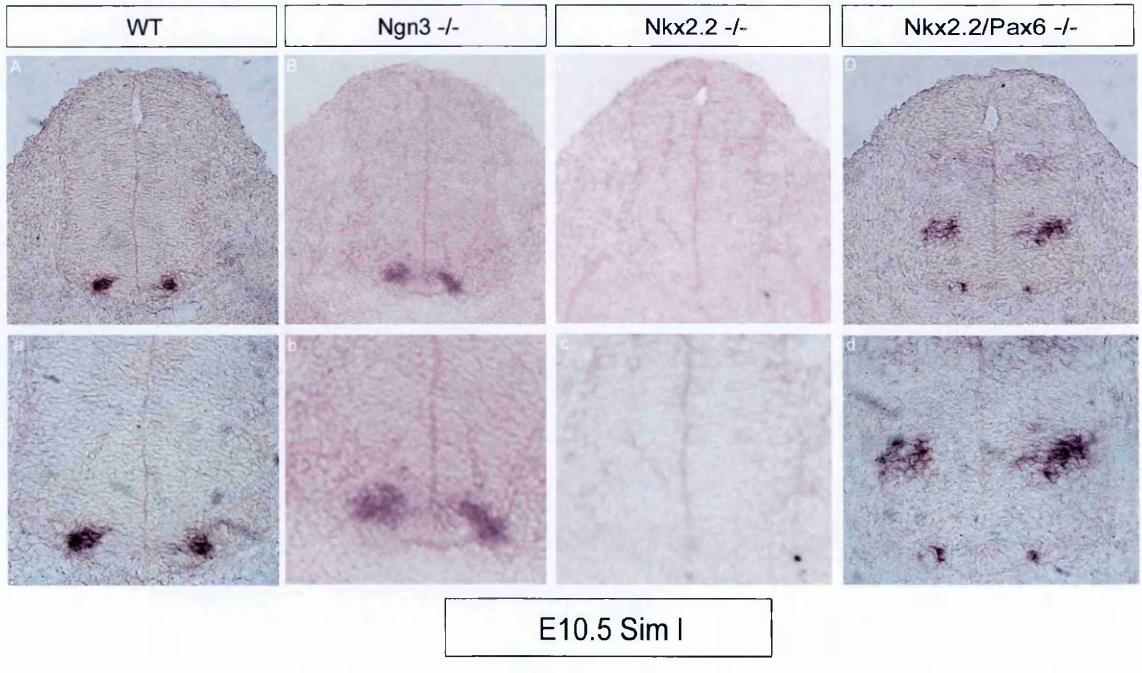

Figure 51 Spinal cord expression of *Sim1* in E10.5 WT, $Ngn3^{-/-}$, $Nkx2.2^{-/-}$and $Nkx2.2/Pax6^{-/-}$ embryos [Dorsal (top) – Ventral (bottom)]

Figure 52: Examination and comparison of the expression patterns of Nkx2.2 in E11.5 WT and Ngn3 mutant embryos showed a normal Nkx2.2 expression in both the WT (C & c) and the mutant (D & d). V2 neuronal markers, Chox10 and Gata3, were also unaffected in Ngn3 (B, b, D & d) embryos when compared to wild type (A, a, C & c) embryos indicating that Ngn3 is not required for V2 neuronal generation (using immunohistochemistry). All spinal cord sections are from the forelimb region of the embryo. [a-d are higher magnifications (x 20) of the upper panels (x10)]

Figure 53: To assess ventral neural tube development in Ngn3 mutants we examined the expression pattern of the MN marker HB9 (using immunohistochemistry). HB9 expression was normal in Ngn3 mutants (B & b) indicating that Ngn3 is not required to define the ventral limit of MN generation. All spinal cord sections are from the forelimb region of the embryo. [a-b are higher magnifications (x 20) of the upper panels (x10)]

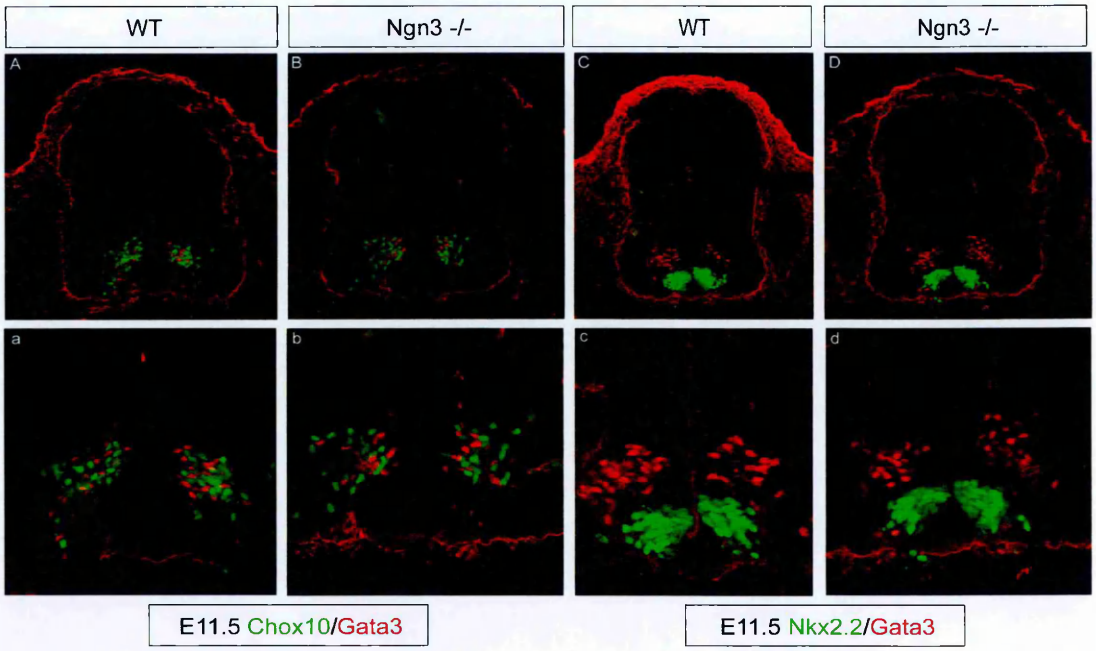

Figure 52 Spinal cord expression of Chox10, Gata3 & Nkx2.2 in E11.5 WT and Ngn3$^{-/-}$ embryos [Dorsal (top) – Ventral (bottom)]

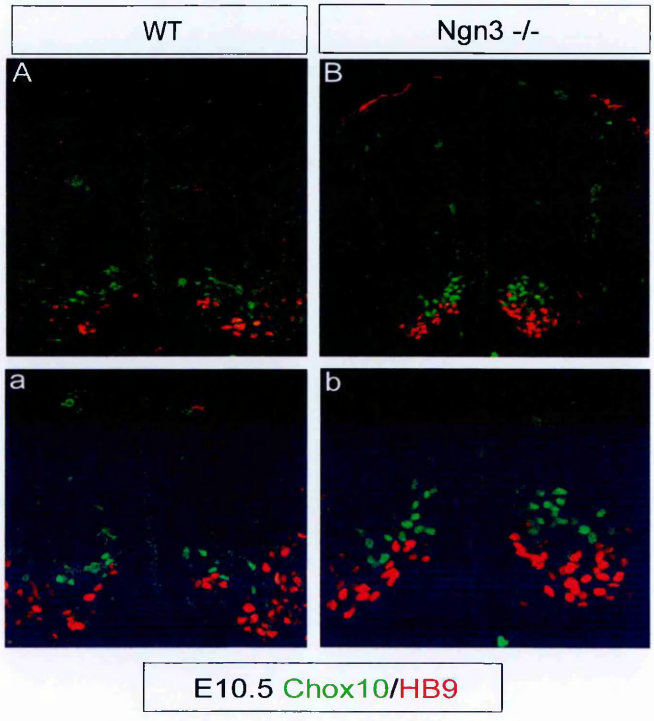

Figure 53 Spinal cord expression of Chox10 & HB9 in E10.5 WT and Ngn3$^{-/-}$ embryos [Dorsal (top) – Ventral (bottom)]

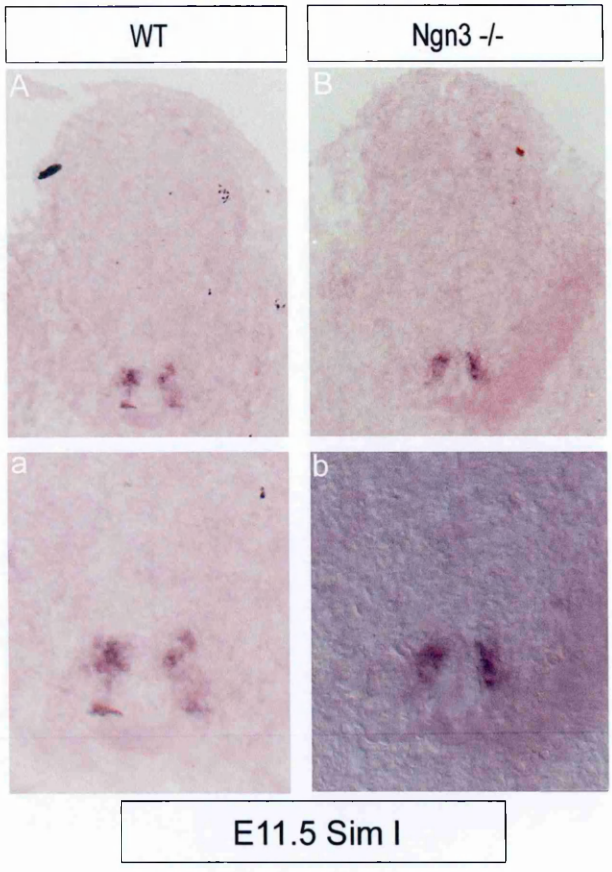

Figure 54 Spinal cord expression of *Sim1* in E11.5 WT and Ngn3$^{-/-}$ embryos [Dorsal (top) – Ventral (bottom)]. The V3 neuronal marker *Sim1*, appears normal in Ngn3$^{-/-}$ mutants (B & b) when compared to WT (A & a) littermates (using *in situ* hybridisation). All spinal cord sections are from the forelimb region of the embryo. [a-b are higher magnifications (x 20) of the upper panels (x10)]

7. Discussion

7.1 The Zebrafish Spinal Cord: A good model for studying dorsal-ventral neural patterning.

In the last decade the zebrafish has received considerable attention as a model for vertebrate development. The isolation of stable mutant and transgenic lines along with studies using morpholino antisense oligonucleotides to disrupt mRNA translation have provided us with insight and information on vertebrate development (Lewis and Eisen, 2003; Udvadia and Linney, 2003). Here, we identified a series of zebrafish neural markers, homologous to the mouse and the chick and we showed that their expression pattern is similar to that seen in other vertebrates. These data create an additional model that can be used for studies on spinal cord patterning.

Similarly to the mouse and the chick, in zebrafish, 24 hpf, Shh expression in the neural tube is confined to the floor plate cells. However, expression of Shh in the notochord is very weak at this stage and is located caudally, in contrast to the mouse and the chick where expression in the notochord is maintained even at later developmental stages (Echelard et al.., 1993). Due to genome duplications in zebrafish two other Hh genes are present; twhh, which is expressed in the floor plate and ehh, which is exclusively expressed in the notochord (Currie and Ingham, 1996; Ekker et al.., 1995). By the end of somitogenesis (24hpf), twhh is still strongly expressed in the floor plate while ehh expression is downregulated but still present in the notochord (Currie and Ingham, 1996; Ekker et al.., 1995).

The expression pattern of progenitor domain markers also correlated to the expression pattern seen in other vertebrates. The paired homeodomain containing transcription factors Pax3 and Pax7 are expressed in the dorsal neural tube while expression of the two Pax6 zebrafish homologues, Pax6.1 and Pax6.2, extends from dorsal to more ventral regions of the spinal cord in a similar manner to that observed in mouse and chick studies. Conversely, the zebrafish Nkx2.2 homologue can be used as a ventral neural tube marker since it was found to be expressed in the same ventral-most progenitor domain of the neural tube, just above the floor plate, similarly to the mouse and chick Nkx2.2 (Barth and Wilson, 1995). Both Nkx6 zebrafish homologues, Nkx6.1 and Nkx6.2, were expressed, with overlapping patterns, in the ventral neural tube in the region where future MNs will emerge and the ventral expression of the zebrafish Olig2 confirms it as marker for the motor neuron progenitor domain (pMN). The zebrafish Axial1 is expressed in the floor plate in the same way as *FoxA2* is in the mouse. Finally, of the three zebrafish Dbx homologues, only Dbx1a expression was observed in the intermediate neural tube in a manner similar to that of mouse Dbx1.

We also identified various neuronal markers and provide evidence that their expression pattern is similar to that previously observed in the mouse and the chick. The zebrafish Pax2.1 expression is located laterally in the intermediate and dorsal neural tube marking post mitotic interneurons, while gata2 and zEvx mark V2 and V0 interneurons respectively. zIslet1, a ventral neural tube marker, can be used as a motorneuron marker since its expression is strongly correlated to that seen in the mouse and the chick (Korzh et al.., 1993).

These data indicate that the overall D-V structure and gene expression profile shares remarkable similarity in vertebrates. Moreover, in *Drosophila* a number of homeobox-containing genes (*vnd*, *ind* and *msh*) display sequence similarity and expression patterns to

vertebrate gene family members (*nkx, gsh and msx*) (Cornell and Ohlen, 2000). Although vertebrates and arthropods show little external similarity it is believed that D-V patterning has been conserved for over 800 million years (Cheesman et al.., 2004; Cornell and Ohlen, 2000). This is supported by the fact that despite the morphological differences seen in the CNS of flies and vertebrates their corresponding spatial CNS domains express homologues genes (Cheesman et al.., 2004; Cornell and Ohlen, 2000).

Together, we identified various ventral, intermediate and dorsal neural tube markers with expression patterns similar to those seen in the mouse and the chick. Due to genome duplications in zebrafish (Postlethwait et al.., 1998) frequently more than one gene ortholog is present compared with non-teleost vertebrates and in most cases at least one, if not both, of the orthologs has a similar expression pattern to the equivalent mouse or chick gene. This is a novel collection of zebrafish progenitor and neuronal markers that share great similarity in their expression profile to the mouse and the chick and create a unique set of reagents to be used for further studies on spinal cord patterning and neuronal induction and specification.

7.2 Hh signalling is necessary for patterning the zebrafish ventral neural tube.

Although HD proteins are conserved in vertebrates and flies, generation, reception and transduction of Hh signals differs significantly (Ingham and McMahon, 2001). Studies from the mouse and the chick have shown that Shh secretion from the notochord and the floor plate is both necessary and sufficient for the induction of most ventral cell types (reviewed by (Briscoe and Ericson, 2001; Jessell, 2000)}). Accordingly, Shh mutant mice fail to generate ventral structures in the CNS and misexpression of Shh can induce floor

plate differentiation *in vitro* (Chiang et al.., 1996; Echelard et al.., 1993). Additionally, recent studies have provided evidence that a group of homeodomain proteins classified as Class I proteins (Pax7, Dbx1, Dbx2 and Pax6) are repressed by Shh signalling at distinct concentration thresholds, while Class II proteins (Nkx2.2) are induced by Shh signalling (Briscoe et al.., 2000).

Previous studies have shown that Hh signalling has a direct role in patterning the vertebrate neural tube and is essential for the specification of all ventral progenitor identities (Wijgerde et al.., 2002). Previous work on *Shh*, *Smo*, and *Gli3* single and double mutants have shown that Hh signalling specifies neural cell identity by negating the repressive action of Gli3 on p0, p1, p2, and pMN formation (Wijgerde et al.., 2002).

Here, we showed that Hh signalling is also necessary for patterning the zebrafish ventral neural tube. In the absence of Hh signalling ventral progenitor and neuronal markers are lost while intermediate markers expand ventrally, possibly because they are no longer repressed by Hh signalling. These results provide evidence that in the absence of Hh signalling ventral neural tube patterning is inhibited in a similar way as in the mouse and the chick.

To block Hh signalling, 10μM of the Shh signalling inhibitor cyclopamine was used. Zebrafish embryos, straight after fertilisation, were incubated for 24 hours in cyclopamine medium. As previously described, all cyclopamine treated embryos showed cyclopia and U-shaped somites (Incardona et al.., 1998; Odenthal et al.., 2000). However, in these cases cyclopia was also observed in the ethanol control treated embryos. Previous cases where ethanol exposure causes cyclopia in fish have been reported (Blader and Strahle, 1998). We established that exposure to ethanol did not interfere with D-V patterning of the neural tube. This was established by analysing the D-V patterning of the

neural tube in ethanol-only treated embryos where the expression pattern of progenitor and neuronal markers remained unchanged.

The expression pattern of progenitor and neuronal markers in cyclopamine treated zebrafish embryos resembled previous observations seen in the mouse and the chick when Shh signalling is missing. The progenitor domain marker *Nkx2.2* and the neuronal marker *Olig2* appear to require Hh signalling for their induction since in embryos treated with cyclopamine these markers were not present in the ventral neural tube. On the other hand, the intermediate marker Dbx expanded ventrally supporting the idea that it is normally repressed by Hh signalling. In most cases the floor plate marker *Axial1* was downregulated but not completely lost. This agrees with previously published data suggesting that, in zebrafish, Hh signalling is required only for the induction of lateral floor plate cells and not all floor plate cells (Odenthal et al.., 2000). *Shh* and *twhh* expression pattern remained unchanged in cyclopamine treated embryos indicating that alterations in the expression of genes in the spinal cord were not due to the loss of expression of hedgehog genes but due to blockade of Hh signalling.

In contrast to ventral and intermediate neural tube markers, which resemble the regulation of their orthologs in other vertebrates, *Pax3* expression was not affected, as expected, in the cyclopamine treated embryos. This was consistent even when a higher concentration of cyclopamine (100μM) was used ensuring that the effect observed was not due to low dosage of cyclopamine. Previous studies on chick embryos, where the notochord was ablated to reduce Shh signalling, have shown ectopic *Pax3* expression in more ventral positions (Goulding et al.., 1993). Similarly, when a notochord graft was placed adjacent to the neural plate it prevented cells adjacent to the implanted graft from expressing *Pax3* (Goulding et al.., 1993). Based on this work Goulding et al.. suggested a model where signals from dorsal cells activate *Pax3* expression while signals from the notochord, in a

distance dependent manner, repress *Pax3* expression (Goulding et al.., 1993). However, the normal *Pax3* expression in the cyclopamine treated zebrafish embryos suggests an alternative model. It is possible that not all dorsal neural tube genes in zebrafish are repressed by Hh signalling in comparison to the amniotes and that some depend solely on the induction by dorsal signals. A future approach, that would provide more understanding on how dorsal markers are specified in zebrafish, is to study signals that derive from the dorsal organiser (e.g. BMPs) and their role in opposing to Hh signals deriving from the ventral organiser.

7.3 Evidence for graded Hh signalling *in vivo*

Shh has been proposed to act as a morphogen providing positional information to ventral progenitor cells over a long distance and in a graded manner in the vertebrate ventral neural tube (Briscoe and Ericson, 2001). For a signalling molecule to be described as a morphogen it must be released from a localised source to form a long range concentration gradient. Progenitor cell populations exposed to different concentrations of the signalling molecule acquire distinct identities. Shh has also been shown to be necessary and sufficient for the induction of distinct neuronal subtypes, both *in vivo* and *in vitro*, at defined positions in the ventral neural tube (Briscoe and Ericson, 2001). Moreover, it has been shown, *in vitro*, that the closer to shh signalling source progenitor cells are located, the higher shh concentration is needed for neuronal induction from these progenitor populations (Ericson et al.., 1997a). In this study we provide evidence for the existence of a gradient of Hh signalling *in vivo*.

Having established the zebrafish spinal cord as a model for studying neural patterning as well as the importance of Hh signalling in patterning the zebrafish ventral

spinal cord we investigated the concentration requirements of Hh signalling in zebrafish. To approach this we first identified the time window necessary for Hh signalling in vivo by blocking Hh signalling at different developmental stages.

When Hh signalling is blocked at early development stages, 1 cell stage to 14 hpf, there is no induction of the ventral neural tube markers *Nkx2.2* and *Olig2* and there is reduced induction of *Nkx6.1*. However, consistent with recently published data, *Nkx6.1* expression even though markedly downregulated is never lost, indicating that although Hh signalling is required for the induction and/or maintenance of the majority of *Nkx6.1* expression some expression persists in the absence of Hh signalling (Cheesman et al.., 2004). Additionally, ectopic expression of *Dbx1* positive cells in more ventral positions was observed when Hh signalling was blocked during the first 12 hours after fertilisation. When Hh signalling was blocked at later developmental stages no effect on the patterning of these markers was observed. The expression pattern of the floor plate marker *Axial1* was also downregulated when Hh signalling was blocked early in development but appeared unaffected when Hh was blocked at later stages (12hpf onwards). This indicated the Hh requirement for the induction of lateral floor plate cells between 1 cell stage and 12 hpf. The expression of the zebrafish Hh genes, shh and twhh, was unaffected no matter the developmental stage Hh signalling was blocked. This confirmed that our observations were indeed due to loss of Hh signalling, mediated by cyclopamine, and not due to the loss of Hh gene expression.

Having defined the window in development where the presence of Hh signalling is necessary for ventral neural patterning to occur, we examined whether the strength of Hh signalling influenced D-V organisation of the spinal cord. Zebrafish embryos, 10 hpf, were transferred to a range of cyclopamine concentrations starting from 10μM to 0.1μM. At high concentrations of cyclopamine (10μM-0.3μM) expression of ventral markers *Nkx2.2* and

Olig2 was lost while *Nkx6.1* was downregulated. *Nkx6.1* and *Olig2* expression was recovered when 0.2μM cyclopamine concentration was used but *Nkx2.2* was only recovered at 0.1μM cyclopamine concentration.

These data indicate that Hh signalling is necessary during a defined developmental period for the induction of ventral neuronal cell fates. If Hh is completely blocked at any developmental stage prior to the pre-determined time of neuronal specification, induction of ventral neuronal subtypes fails to proceed normally. Moreover these data support the idea that different ventral neural tube markers, *Nkx6.1*, *Nkx2.2* and *Olig2* respond to different concentrations of Hh signalling. The concentration of cyclopamine to which a ventral marker is sensitive corresponds to the distance from the source of Hh. The closer to the signalling source a ventral marker is expressed the more sensitive it is to cyclopamine. This supports the idea that Hh signalling functions in a graded manner to control gene expression in the ventral neural tube.

7.4 Duration of Hh signalling also influences the D-V organisation of the zebrafish spinal cord.

Our data indicate that in addition to the concentration of Hh signalling, genes in the ventral neural tube are differentially sensitive to the duration of Hh signalling. Expression of *Nkx2.2* and *Olig2* was lost in the spinal cord of embryos placed into cyclopamine from 1 cell stage to 14 hpf. Similarly, *Nkx6.1* expression was downregulated in the spinal cord of cyclopamine treated embryos during the same time-scale. Conversely, if embryos were transferred into cyclopamine 18 hpf or later expression of all three ventral markers appeared normal. If embryos were transferred into cyclopamine 16hpf expression of *Nkx6.1*

was normal, expression of *Olig2* was partially recovered but expression of *Nkx2.2* still appeared severely downregulated and only a few *Nkx2.2* positive cells were present. If embryos were transferred into cyclopamine at any later stage, 18 hpf onwards, then all three ventral gene expression appeared normal. This observation indicated that ventral genes respond differentially to the time of exposure to Hh signals.

Together these data suggest that cells respond to Hh by integrating signalling strength over time rather than responding at a defined time point to a pre-established ligand gradient. Thus both the duration and strength of signalling appear to control D-V patterning of the ventral neural tube. Previous studies have suggested a model termed 'sequential cell context' that could provide a molecular mechanism that underlies these observations (Pages and Kerridge, 2000). According to this model initial exposure to signalling changes cell context, which in combination with continuing morphogen activity, results in the expression of novel targets (Pages and Kerridge, 2000). This model could explain how the cross-repressive interactions between Class I (Shh repressed e.g. Pax6) and Class II (Shh induced e.g. Nkx2.2) HD proteins are used to achieve D-V patterning. Early exposure of progenitor cells to Shh signalling could cause downregulation of Pax6 providing the necessary cell context for the induction of Nkx2.2 once prolonged signalling has been received.

7.5 Neuronal induction and specification in the ventral neural tube of Pax6/Nkx2.2 double mutant embryos.

Graded Shh signalling establishes D-V positional identity by regulating HD gene expression in neural progenitors. The sharp boundaries of the individual progenitor domains are maintained due to the cross-repressive interactions between Class I (Shh

repressed) and Class II (Shh induced) HD proteins (Briscoe et al.., 2000). Interaction between Pax6 (Class I) and Nkx2.2 (Class II) proteins defines and maintains the pMN/p3 boundary (Briscoe et al.., 2000).The analysis of the $Pax6^{-/-}$ and $Nkx2.2^{-/-}$ mutants, has provided us with valuable information in understanding the patterning of the ventral neural tube and has given us insight into how specific progenitor cell populations are programmed to give rise to distinct neuronal subtypes. Both Nkx2.2 and Pax6 genes have been shown to have significant roles in ventral neuronal patterning and a critical role in interpreting graded Shh signals that derive from the notochord and the floor plate (Briscoe et al.., 1999; Ericson et al.., 1997b).

Elimination of Nkx2.2 results, at spinal cord level, in a ventral-to-dorsal transformation of ventral progenitor cells while conversely, Pax6 elimination, forces ventral progenitor cells to undergo a dorsal-to-ventral transformation (Briscoe et al.., 1999; Ericson et al.., 1997b). In the spinal cord of Nkx2.2 mutants, motor neurons are generated from the ventral-most progenitor domain at the expense of V3 interneurons while in the Pax6 mutants V3 neurons expand dorsally at the expense of MN generation (Briscoe et al.., 1999; Ericson et al.., 1997b). In the hindbrain, visceral motor neurons are generated instead of V3 interneurons (Briscoe et al.., 1999). In contrast to the observations in the spinal cord, in the absence of Nkx2.2, vMNs are maintained in the p3 domain and it has been suggested that the presence of Nkx2.9, an Nkx2.2 related gene, still present within the p3 domain accounts for the identity domain maintenance (Briscoe et al.., 1999). Similarly to the spinal cord observations, in Pax6 mutants vMNs expand dorsally in expense of MN generation (Briscoe et al.., 1999; Ericson et al.., 1997b).

Here, we generated Nkx2.2/Pax6 double mutants and subsequently analysed the generation of ventral neuronal subtypes. Based on the work done on the single mutants we created a model predicting the expression pattern of ventral neuronal and progenitor

markers in the spinal cord of Nkx2.2/Pax6$^{-/-}$ embryos (Fig. 29). According to this model, in the ventral spinal cord of E10.5 double mutant embryos the absence of Nkx2.2 should result in MN generation from the ventral-most progenitor domain and in the subsequent loss of V3 interneurons normally generated from that domain. We would expect that V2 interneurons are generated normally while Pax6 loss results in the loss of V1. The phenotype of the double mutant embryos should resemble that of Nkx2.2 mutants at spinal cord levels (Fig. 29).

The loss of the V3 interneuron markers, Ngn3 and Sim1, from the ventral-most spinal cord progenitor domain of the Nkx2.2/Pax6$^{-/-}$ embryos indicates that V3 neurons fail to be generated in double mutant embryos in the ventral-most spinal cord region, which is in agreement to our predicted model. Additionally, presence of ectopic motor neuron markers, Isl1/2, HB9 and Olig2, within the p3 domain confirms MN generation in more ventral domains that result in the loss of V3 interneurons.

Unexpectedly, Sim1 expression was also detected dorsally in the spinal cord of double mutant embryos in a similar fashion to the ectopic dorsal Sim1 expression previously reported in Pax6 mutants (Ericson et al.., 1997b). This suggests that Pax6 may be involved in the repression of V3 interneuron generation. Moreover, the expression of Sim1 in the absence of Nkx2.2 indicates that Nkx2.2 is not strictly required for the generation of Sim1 expressing cells. Additionally, the absence of ectopic Sim1 within the pMN domain suggests the presence of another molecule responsible for acting as Sim1 repressor. Motor neuron marker Olig2 is maintained within the pMN domain and expands ventrally in the absence of Nkx2.2. This is the region where Sim1 is excluded making Olig2 a candidate molecule responsible for repressing V3 generation. Therefore, Olig2 expansion ventrally and not absence of Nkx2.2, as previously suggested (Briscoe et al.., 1999), may be the reason that V3 neurons are lost in Nkx2.2$^{-/-}$ embryos. Based on these

results we propose a possible model where Sim1 expression is repressed by Pax6 and Olig2 (Fig. 54). Finally, V2 neuronal markers, Chox10, Gata3 and FoxD3, are downregulated in double mutant embryos, in the domain where dorsal ectopic Sim1 is detected, presumably due to generation of V3 interneurons in that region.

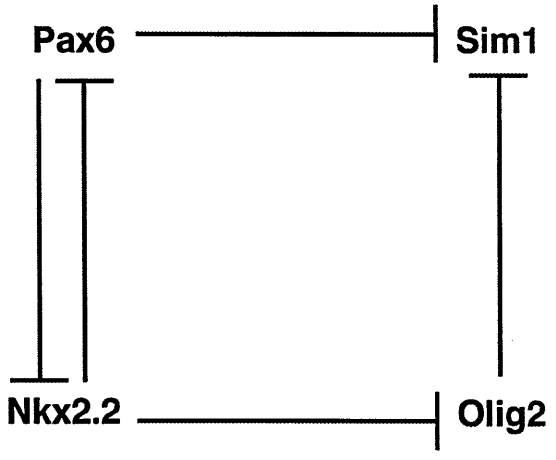

Figure 55 Diagram proposing a model where expression of Sim1 is repressed by Pax6 and Olig2.

7.6 Specification of the hindbrain neuronal subtypes in Nkx2.2/Pax6$^{-/-}$ double mutants.

Previous studies have indicated that ventral neuronal patterning at spinal cord and hindbrain levels differs significantly in Nkx2.2 mutant animals. At hindbrain levels the ventral-most progenitor domain gives rise to visceral motor neurons instead of V3 interneurons (Ericson et al.., 1997b). Most importantly, in Nkx2.2 mutants hindbrain visceral motor neurons and somatic continue to be generated normally and there is no ventral-to-dorsal switch corresponds to that observed in the spinal cord of these animals (Briscoe et al.., 1999). It has been suggested that it is the redundant activity of Nkx2.9, a gene closely related to Nkx2.2, still present in the hindbrain of Nkx2.2 mutants that is

responsible for the normal generation of visceral motor neurons and the exclusion of somatic MNs from that domain (Briscoe et al.., 1999).

Based on previous studies on the hindbrain of both Nkx2.2 and Pax6 mutants, we predicted the consequences on neuronal induction and specification in the hindbrain of Nkx2.2/Pax6 double mutant animals (Fig. 30). This prediction suggests that the hindbrain phenotype of Nkx2.2/Pax6$^{-/-}$ embryos would be most similar to the hindbrain phenotype of Pax6$^{-/-}$ embryos where expansion of Nkx2.9 dorsally due to the absence of Pax6 should prevent somatic MN generation in the pMN domain and be sufficient to induce visceral MNs. Additionally, the hindbrain of double mutant embryos should show loss of V1 interneurons, due to the absence of Pax6, along with repression of V2 interneurons, due to Nkx2.9 expansion dorsally.

To our surprise, Nkx2.9 did not expand dorsally as expected due to the loss of Pax6, as observed in Pax6 mutants. The fact that Nkx2.9 did not expand dorsally is apparently associated with the failure of visceral motor neurons to expand dorsally in the pMN domain as confirmed by the normal Phox2B expression in the hindbrain of double mutants. Additionally, the loss of HB9 and Olig2 expression in the hindbrain of double mutant embryos suggests loss of somatic MNs, a phenotype similar to the hindbrain phenotype of Pax6 mutant embryos. The loss of Olig2 in the hindbrain of double mutant embryos in contrast to its expansion at spinal cord levels indicates that Olig2 is regulated differently in the spinal cord and the hindbrain.

V2 interneuron markers, Chox10 and Gata3, were not reduced in double mutant embryos and may be generated in increased numbers. A possible explanation for this is that even though somatic motor neurons are lost in the absence of Pax6 there is no dorsal expansion of visceral motor neurons within the somatic motor neuron progenitor domain and instead these progenitors generate V2 interneurons.

7.7 Presence of Nkx2.2 is required for Ngn3 expression in the spinal cord.

The bHLH transcription factor Ngn3 is expressed during early development in the ventral neural tube and its expression overlaps with Nkx2.2 expression (Briscoe et al.., 1999; Lee et al.., 2003). However, the role of Ngn3 during early development has not yet been established. Studies on Nkx2.2$^{-/-}$ mutants have suggested that Ngn3 may be a downstream mediator of Nkx2.2 since in the absence of Nkx2.2, Ngn3 expression is lost. More recent studies on Ngn3$^{-/-}$ mutants have reported loss of Nkx2.2 expression (Lee et al.., 2003) raising the possibility that Ngn3 and Nkx2.2 expression depend on each other. Reports suggest that in both mutants expression of the V3 interneuron marker Sim1 was disrupted (Briscoe et al.., 1999; Lee et al.., 2003). However, in contrast to Nkx2.2$^{-/-}$ embryos, in Ngn3 mutants MNs do not expand ventrally and there is no evidence of a ventral-to dorsal transformation in the identity of ventrally located progenitor cells (Lee et al.., 2003).

Consistent with previous studies Ngn3 expression was absent in Nkx2.2 mutants (Briscoe et al.., 1999). Moreover, Ngn3 expression was also lost in Nkx2.2/Pax6 double mutants. Conversely, Nkx2.2 expression was maintained in Ngn3 mutants, an observation that contrasts with previously published data that reported Nkx2.2 to be downregulated in Ngn3 mutant embryos (Lee et al.., 2003). Taken together these data indicate that Nkx2.2 is upstream and required for Ngn3 expression in the ventral-most progenitor domain.

To establish the role of Ngn3 in the ventral neural tube patterning we also looked at V3 interneuron marker Sim1. In contrast to previously published data, Sim1 expression was still present in Ngn3 mutants (Lee et al.., 2003). Previous work analysed Sim1 expression in older embryos (E13.5-E14) indicating that while Ngn3 is not required for the induction of V3 interneurons it may be associated with the maintenance of Sim1 expression.

Additionally, the normal expression of MN (HB9) and V2 interneuron (Chox10 & Gata3) markers in Ngn3 mutants suggests that Ngn3 is not required for MN and V2 interneuron induction.

7.8 Conclusions

In this study we demonstrated that zebrafish, similar to the mouse and the chick, can be used as a model for vertebrate spinal cord developmental studies. Gene expression patterning of the ventral zebrafish neural tube is dependent on Hh signalling that derive from the organising centres, notochord and floor plate. In the absence of Hh signalling ventral neural tube patterning is disrupted as shown by the loss of ventrally expressed markers. Our results showed that Hh signalling is necessary for a defined period during early developmental stages to induce ventral neuronal subtypes. Additionally, we showed that ventral neural tube markers are differentially sensitive to the strength and duration of Hh signal *in vivo*. These data provide *in vivo* support for a gradient of Hh signalling that is responsible for providing positional information in the ventral neural tube.

To expand our knowledge on ventral neural tube patterning we generated and analysed the spinal cord and hindbrain of Nkx2.2/Pax6$^{-/-}$ double mutant embryos since analysis of each of the single mutants (Nkx2.2$^{-/-}$ and Pax6$^{-/-}$) has shown that both genes are involved with ventral vertebrate neural patterning. Analysis of the Nkx2.2/Pax6$^{-/-}$ mutant has provided us with more detailed information on how progenitor cell populations perceive information from the organising centres and how they influence neuronal induction and specification. This analysis indicates that Nkx2.2 is not directly required for V3 interneuron generation but instead is required to repress Olig2 or similarly expressed gene. It also appeared that Olig2 is regulated differently at hindbrain and spinal cord levels.

Additionally, Pax6 and Olig2 act by repressing V3 interneuron generation while at hindbrain levels Nkx2.2 is required for the expression of vMNs.

Finally, by analysing $Ngn3^{\Delta/-}$ mutants we examined the function of the two closely related genes Ngn3 and Nkx2.2 and we suggested that Nkx2.2 acts upstream of Ngn3 and is required for Ngn3 expression.

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